Inhibition of the main protease of SARS-CoV-2 (M^{pro}) by repurposing/designing drug-like substances and utilizing nature's toolbox of bioactive compounds

Io Antonopoulou ¹,⇑, Eleftheria Sapountzaki ¹, Ulrika Rova, Paul Christakopoulos

Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental and Natural Resources Engineering, Luleå University of Technology, SE-97187 Luleå, Sweden

Abstract

The emergence of the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) has resulted in a long pandemic, with numerous cases and victims worldwide and enormous consequences on social and economic life. Although vaccinations have proceeded and provide a valuable shield against the virus, the approved drugs are limited and it is crucial that further ways to combat infection are developed, that can also act against potential mutations. The main protease (M^{pro}) of the virus is an appealing target for the development of inhibitors, due to its importance in the viral life cycle and its high conservation among different coronaviruses. Several compounds have shown inhibitory potential against M^{pro}, both in silico and in vitro, with few of them also having entered clinical trials. These candidates include: known drugs that have been repurposed, molecules specifically designed based on the natural substrate of the protease or on structural moieties that have shown high binding affinity to the protease active site, as well as naturally derived compounds, either isolated or in plant extracts. The aim of this work is to collectively present the results of research regarding M^{pro} inhibitors to date, focusing on the function of the compounds found by in silico simulations and further explored by in vitro and in vivo assays. Creating an extended portfolio of promising compounds that may block viral replication by inhibiting M^{pro} and by understanding involved structure–activity relationships, could provide a basis for the development of effective solutions against SARS-CoV-2 and future related outbreaks.

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⇑ Corresponding author. 
E-mail address: io.antonopoulou@ltu.se (I. Antonopoulou).
¹ Equal contribution.

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1. Introduction

As of the beginning of 2020, the world is going through a pandemic, which apart from a severe public health crisis counting >219 million cases and >4.5 million deaths, has had a tremendous impact on economic and social life. In December 2019, in the city of Wuhan, Hubei province, China, a series of pneumonia cases were reported, exhibiting symptoms such as fever, dry cough, chest discomfort or even dyspnea and bilateral lung infiltration. Further investigation led to the identification of a novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), as the responsible pathogen. The disease caused by the virus, was named as COVID-19 (Coronavirus disease 2019) and was widely spread all over the world, resulting in the World Health Organization (WHO) declaring a pandemic on 11 March 2020 [1,2]. SARS-CoV-2 is the third coronavirus creating a public health concern in the past 20 years, after the severe acute respiratory syndrome-coronavirus (SARS-CoV) and Middle East respiratory syndrome (MERS-CoV), which created an outbreak in 2002 and 2012, respectively. SARS-CoV-2 shares common genomic sequence by a percentage of 79% with SARS-CoV and 50% with MERS [3].

Therapeutic targets to combat COVID-19 include structural and functional proteins of the virus, as well as virulence factors and host proteins that are useful for viral proliferation. So far, only remdesivir, an inhibitor of the RNA dependent RNA polymerase of the virus, has been FDA-approved for use in COVID-19 patients [4], while some monoclonal antibody treatments have received authorizations for emergency use [5].

The translation of the viral RNA of SARS-CoV-2, once it enters the host cells, leads to the synthesis of two polyproteins, pp1a and pp1ab. After auto-processing its own N- and C- terminals to the host cells, leads to the synthesis of two polyproteins, pp1a and pp1ab, catalyzing the formation of nonstructural proteins necessary for the construction of the replication transcription complex that the virus needs in order to synthesize new RNA [6–8]. The proteolysis takes place in >11 cleavage sites. The amino acid sequence that the enzyme recognizes as a cleavage site is (Leu-Gln)-(Ser/Ala/Gly), with the peptide bond being hydrolyzed after Gln. The vital role of M*pro in the reproduction of SARS-CoV-2 and the release of many of its proteins, combined with the fact that its structure and mechanism have been investigated, make it a very appealing target to block viral activity. Moreover, the fact that there is no human enzyme cleaving proteins after the Gln residue is another advantage of M*pro as target for the development of inhibitors to act as antiviral drugs or immune-boosting compounds, as it increases its specificity and limits unwanted side effects. Lastly, the high conservation of the protease among coronaviruses, depicted by the high amino acid sequence identity (96% sequence identity between SARS-CoV and SARS-CoV-2 main proteases), is another factor that implies that the development of M*pro inhibitors can be useful for different SARS-CoV-2 strains and mutants or future coronavirus outbreaks [9–15].

The present work is a collective presentation of the existing research results regarding potential inhibitors of the major functional protein of SARS-CoV-2, M*pro, including drug-like and natural compounds that have been investigated in silico and in vitro. Recent developments for compounds that have been selected for in vivo and clinical trials are also discussed, highlighting the importance of M*pro as target among the recurring virus mutants. In particular, the impressive number of published research during the past 2 years on proposing novel solutions for M*pro inhibition highlights the need for complementary measures to vaccination and medication strategies, such as developing functional aids that can help in boosting immunity and aid protection against infections by coronaviruses.

2. The main protease of SARS-CoV-2 (M*pro)

SARS-CoV-2 M*pro is a cysteine protease (EC 3.4.22.69) and a member of the PA clan of proteases. Proteases are enzymes that hydrolyze peptide bonds and thus belong to the category of hydrodases. The first crystal structure of SARS-CoV-2 M*pro was determined by X-ray diffraction at a resolution of 2.16 Å and was deposited at the Protein Data Bank (PDB) by Jin et al. and released on February 5, 2020, under the PDB ID 6LU7 [7]. Since then, many structures of the protease have been deposited, including the enzyme co-crystallized with various inhibitors. The active form of the enzyme is a homodimer (Fig. 1). The structure of a single monomer consists of a 306-residue-long polypeptide chain, which can be divided into three domains: domain I (residues 8–101), domain II (residues 102–184) and domain III (residues 201–303). Domains I and II are composed of antiparallel β-barrels and host the active site in a cleft formed between them, whereas domain III consists of 5 α-helices and plays a role in the dimerization of the enzyme. Residues 185–200 form a loop that connects domains II and III [7,15,16]. The enzyme is active only as a dimer because developments for compounds that have been selected for in vivo and clinical trials are also discussed, highlighting the importance of M*pro as target among the recurring virus mutants. In particular, the impressive number of published research during the past 2 years on proposing novel solutions for M*pro inhibition highlights the need for complementary measures to vaccination and medication strategies, such as developing functional aids that can help in boosting immunity and aid protection against infections by coronaviruses.

Fig. 1. SARS-CoV-2 M*pro in the active form of a homodimer (PDB ID:7JKV). The right monomer is shown as surface while the left monomer portrays the secondary structure and the three domains of the enzyme. Domain I is in red, domain II in purple and domain III in cyan. Catalytic residues His41 and Cys145 are highlighted in yellow and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the NH₂-terminal of each protomer interacts with residue Glu166 of the other protomer and contributes to the formation of the S1 subsite of active site [17]. Due to this interaction, the NH₂-terminal of a monomer is positioned between domains II and III of this monomer and domain II of the other monomer. The dimeric structure of the enzyme is regulated through a salt-bridge between residues Glu 290 of one protomer and Arg4 of the other [15]. At its active site, the enzyme has a cysteine-histidine catalytic dyad (Cys145-His41). The existence of the stabilizing oxyanion hole, consisting of residues Gly143, Ser144 and Cys145, is also noteworthy. During catalysis, the negative charge of the carbonyl oxygen in the scissile bond of the natural substrate of the protease is being balanced by the oxyanion hole. It is also reported that the oxyanion hole similarly stabilizes inhibitors, as many of them form a hemithioacetal intermediate with a negatively charged oxygen atom and bind to the Cys145 residue of the protease with a similar geometry as the tetrahedral intermediate formed by the natural substrate [10,13,15]. Except for the catalytic dyad (Cys145, His41), the active site of M pro is demarcated by residues Ser46, Gln189, Thr190, Ala191, Pro168, Glu166, Leu141 and Asn142 [16]. It consists of four main subsites, S1, S1', S2 and S4, similar to the active sites of the main proteases of other coronaviruses [9,18]. More specifically, out of the 306 residues of the protease sequence, only 12 are different between the main proteases of SARS-CoV-2 and SARS-CoV, which corresponds to 96% identity [19].

The proposed catalytic mechanism of the enzyme is based on a reaction of nucleophilic addition (Fig. 2). The cleavage of the peptide bond is suggested to be initiated by a proton transfer from the thiol group of Cys145 to the imidazole of His41. Then, a highly reactive nucleophile ion pair is formed. The Cys residue attacks the carbonyl portion of the scissile peptide bond, forming a thiohemiketal intermediate, while the protonated His attacks the N-atom of the peptide bond, creating the acyl-enzyme complex intermediate. A polypeptide chain is released as the first product of the reaction. Then, an active water molecule attacks the carbonyl carbon atom of the Gln residue, whereas His is being reprotonated, no longer maintaining the acyl-enzyme complex. Lastly, Cys145 is released, as the covalent bond with the peptide is broken. The water molecule taking part in the above series of reactions is also part of interactions between residues His41, His164 and Asp187, balancing the polar contacts between them. Kneller et al. have pointed out its role, characterizing it a part of a potential non-canonical catalytic triad [10].

3. Desired characteristics of SARS-CoV-2 M pro inhibitors

In search of additional therapeutic routes, various compounds have been investigated for their ability to inhibit M pro, including repurposed drugs or other coronavirus’ main protease inhibitors, designed and optimized drug molecules, as well as natural compounds. Inhibition can occur through covalent binding of the inhibitor to the catalytic cysteine, through a mechanism of nucleophilic addition. In this case, the inhibitor often mimics the natural peptide substrate of the enzyme. Although such molecules have higher specificity towards the protease, their pharmacokinetic properties might pose a hindrance to their use as pharmaceuticals. There is also the possibility of non-covalent, reversible inhibitors, which usually have better pharmacokinetic properties and can be more efficiently used as drugs. However, it is more challenging to develop a non-covalent inhibitor, since the structure-activity relationship and the interactions with the protease, which lead to effective inhibition, are not based on the already available information provided by the natural substrate binding and the mechanism of the protease, as it happens in the case of peptide-like, covalent inhibitors. In the case of irreversible inhibitors, the design might be easier but the risk of toxicity due to low selectivity is concerning [20]. In order to establish the interactions that are required with the active site residues to consider a compound as inhibitor, a molecular dynamics study involved different inhibitors in complex with M pro was performed and revealed that Glu166, His41, Gly143, Ser144 and Cys145 are major interacting residues [14].

In the case of covalent peptidomimetic inhibitors, a common way of approaching their structural analysis is through the system of nomenclature for the peptide substrates of proteases. According to this, substrate residues are numbered, beginning from the scissile bond, as P1', P2' etc., to the direction of the C-terminus and as P1', P2 etc. to the direction of the N-terminus (Fig. 3). The catalytic residues are located between S1 and S1' subsites, so that they are accessible by the scissile bond [21]. Several inhibitors have been designed having a glutamine analog at the P1 position, but
research has provided indications that different, hydrophobic moieties can be used in this position [17]. This review includes various studies that have explored the effect of different functional groups in different positions, as well as the potency of different warheads in forming a covalent bond with the catalytic cysteine. An overview of the reported drug-like compounds to date demonstrated as inhibitors of Mpro is presented in Table 1. The inhibitors are categorized as covalent, non-covalent, allosteric, and inhibitors with non-specific binding mode.

4. Repurposed drugs and designed drug-like compounds as inhibitors of Mpro

4.1. Covalent Mpro inhibitors

Research has led to the identification of multiple compounds as Mpro inhibitors, which include both already known drugs, as well as compounds designed for the specific target. The co-crystallization structure of the inhibitors in complex with the enzyme proves that the majority of identified inhibitors bind covalently to the active site. The most dominant strategy in the design of such compounds is mimicking the native peptide substrate of the enzyme, and screening different functional groups to achieve the most favorable interactions. However, several smaller compounds have also been investigated. As mentioned above, due to the high conservation of the active site of the main proteases of various coronaviruses, many already tested inhibitors for SARS-CoV or other coronaviruses are also investigated against SARS-CoV-2.

4.1.1. Peptidomimetic inhibitors with a γ-lactam moiety in the P1 position

A common characteristic among numerous covalent inhibitors is the presence of a γ-lactam group in the P1 position. The carbonyl and the –NH groups of the lactam ring allow the formation of hydrogen bonds in the S1 subsite of the protease, therefore contributing to the reinforcement of the binding of the inhibitor. Most of these inhibitors also possess a carbonyl warhead, either as an aldehyde group or as part of a larger moiety, while they often have a tert-butyl group or another hydrophobic group in the P2 position.

N3 is such compound that successfully inhibits the protease, as it binds to its active site very similarly to the natural substrate. It is the most widely accepted inhibitor in literature, and the one most often used as a reference to evaluate the inhibitory effect of other compounds. It is a Michael acceptor, and acts as a time-dependent, irreversible inhibitor. Its 50% cytotoxicity concentration (CC50) is reported to be 133 μM, whereas the half-maximal effective concentration (EC50) is 16.77 μM. In the original publication that provided the crystal structure, the interactions between the enzyme and N3 are described in detail. More specifically, the inhibitor forms a 1.8 Å covalent bond with the sulfur atom of residue Cys145 of the protein. Moreover, N3 forms one hydrogen bond with each one of residues Gly143, His163, His164, Glu189 and Thr190 and two hydrogen bonds with Glu166 [7].

GC376 is a broad-spectrum antiviral compound, which is also often used as a reference for the evaluation of other potential inhibitors, due to its inhibitory potency and successful prevention of coronavirus infections in animals which sets a direction for clinical trials in humans [22]. It has a half-maximal effective concentration (EC50) of 0.70 μM against SARS-CoV-2, which is very close to the approved anti-SARS-CoV-2 drug remdesivir (EC50 = 0.58 μM). In order for GC376 to form a covalent bond, its bisulfite group is removed. The compound forms one hydrogen bond with residues Phe140, Gly143, Cys145, His163, His164 and two with Glu166. It also interacts with the hydrophobic pocket residues Arg40, His41, Met49, Tyr54 and Asp187 [23]. Effective against SARS-CoV-2 is the parent compound of GC376, GC373. It shows no toxicity in cell culture and inhibits Mpro with a half-maximal inhibitory concentration (IC50) value of 0.40 μM. The inhibition occurs through a reversible reaction of the thiol of Cys145 with the carbonyl of GC373 resulting in a hemithioacetal. The conformation of the inhibitor in the active site is stabilized with hydrogen bonds with the oxyanion hole residues Gly143, Ser144, Cys145. There is also one hydrogen bond formed with His163 and two with Glu166. There are also hydrophobic interactions present, both with S2 subsite residues His41, Met49 and S1 subsite residues Met165 and His172 [24].

Various derivatives exploring the potential of different substitutions in the P2 and P3 positions have been investigated in a study by Vuong et al. [24], where the compounds with the bisulfite moiety (similar to GC376) showed better inhibitory potency compared to the respective aldehydes (such as GC373). The derivatives that stand out are inhibitors 2c and 2d, where a cyclopropyl group has been introduced in the P2 position of both inhibitors, as it was proven to be the most favorable substitution and a 3-fluorobenzyl or a 3-chlorophenylethyl moiety, respectively, took

Fig. 3. Proteolytic enzyme substrate nomenclature. S2, P2 is marked in purple, S1-P1 in green, S1-P1 in red and S2-P2 in brown (left). Example of the binding of inhibitor N3 in the active site of Mpro (right). The residues that form each subsite, as described by [3], are shown in the respective colors. The light colors correspond to residues that contribute with their backbone to the formation of the subsite, while the darker colors to the ones that contribute with their side chain. The residues depicted in two colors stand out are inhibitors 2c and 2d, where a cyclopropyl group has been introduced in the P2 position of both inhibitors, as it was proven to be the most favorable substitution and a 3-fluorobenzyl or a 3-chlorophenylethyl moiety, respectively, took

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Table 1
Drug-like compounds with inhibitory effect against SARS-CoV-2 M<sub>pro</sub> and their inhibitory properties.

| Name                  | PDB ID       | H-bonds                                                                 | IC<sub>50</sub> (µM) | Calculation method              | EC<sub>50</sub> (µM) | Calculation method              | CC<sub>50</sub> (µM) | Calculation method              | Reference |
|-----------------------|--------------|--------------------------------------------------------------------------|------------------------|---------------------------------|------------------------|---------------------------------|----------------------|---------------------------------|-----------|
| Covalent inhibitors   |              |                                                                          |                        |                                 |                        |                                 |                      |                                 |           |
| N3                    | 6LU7         | Gly143, His163, His164, Glu166, Gln189, Thr190                          | –                      | –                               | –                      | 16.77                           | Plaque reduction assay       | 133 MTS cell proliferation assay in Vero E6 cells | [7]       |
| GC376                 | 7D1M         | Phe140, Gly143, Cys145, His163, His164, Glu166                          | 0.19                   | FRET–based assay                 | 0.92                   | Plaque reduction assay          | >200 CellTiter-Glo assay in Vero E6 cells | [24]      |
| GC373                 | 6WTK         | Gly143, Ser144, Cys145, His163, Glu166                                  | 0.4                    | –                               | 1.5                    | –                               | >200 CellTiter-Glo assay in Vero E6 cells | [24]      |
| Compound 2c           | –            | Not described                                                            | 0.07                   | –                               | 0.57                   | –                               | >200 CellTiter-Glo assay in Vero E6 cells | [24]      |
| Compound 2d           | –            | Not described                                                            | 0.08                   | –                               | 0.7                    | –                               | >200 CellTiter-Glo assay in Vero E6 cells | [24]      |
| Compound 2            | 7KOE         | Phe140, His163, His164, Glu166, Gln189                                  | 0.18                   | FRET–based assay                 | 0.086/0.069            | Antiviral assay in Vero E6/ A549<sup>ACE2</sup> cells | >100 Cytotoxicity assay in Vero E6 / CRFK cells | [25]      |
| MPII                  | 7IPZ         | Asn142, Cys145, His163, Met165, Glu166, Gln189                          | 0.10                   | Fluorescent peptide assay        | >10                    | Virus-based microneutralization assay in Vero E6 cells | – –                  | [26]      |
| MPI3                  | 7JQ0         | Asn142, Cys145, His163, Met165, Glu166, Gln189                          | 0.0085                 | –                               | >10                    | Virus-based microneutralization assay in Vero E6 cells | – –                  | [26]      |
| MPI5                  | 7JQ2         | Not described                                                            | 0.033                  | –                               | 5/0.16–0.31             | Virus-based microneutralization assay in Vero E6/E549<sup>ACE2</sup> cells | – –                  | [26]      |
| 11a                   | 6LZE         | Cys145, His163, His164, Glu166                                         | 0.053                  | FRET–based assay                 | 0.53                   | Plaque reduction assay          | – –                  | [9]       |
| 11b                   | 6M0K         | Cys145, His163, His164, Glu166                                         | 0.04                   | –                               | 0.72                   | –                               | – –                  | [9]       |
| UAWJ9-36–1            | 7LYH         | Phe140, Asn142, Gly143, His163, Glu166                                  | 0.051                  | FRET–based assay                 | –                      | –                               | – –                  | [27]      |
| UAWJ9-36–3            | 7LYI         | Phe140, Asn142, Gly143, His163, Glu166                                  | 0.054                  | –                               | –                      | –                               | – –                  | [27]      |
| MI-23                 | 7D3I         | Phe140, Gly143, Cys145, His163, His164, Glu166                          | 0.0076                 | FRET-based assay                 | –                      | >500 CCK8 assay                 | – –                  | [18]      |
| PF-00835231           | –            | His163, His164, Glu166                                                  | 0.221/0.184            | –                               | 2.5/0.16–0.31            | Virus-based microneutralization assay in Vero E6/E549<sup>ACE2</sup> cells | – –                  | [29,53]  |
| PF-07321332           | –            | His163, Glu166, Gln189                                                  | 0.0745/0.0779          | CPE assay in Vero E6 cells/ Nanoluciferase reporter virus assay in A549<sup>ACE2</sup> cells | >100 / >3 | –                               | >100 Cytotoxicity assay in Vero E6 / A549<sup>ACE2</sup> cells | [30]      |
| 5 h (YH-53)           | 7JKV/        | Gly143, Cys145, His164, Glu166, Gln189                                  | 0.0347                 | Fluorogenic substrate enzyme inhibition assay | 4.2                    | RNA-qPCR quantitative assay in VeroE6 cells | >100 RNA-qPCR quantitative assay in VeroE6 cells | [32,33]  |
|                      | 7E18         |                                                                        |                        |                                 |                        |                                 |                      |                                 |           |
| SH-5                  | 7E19         | His41, Gly143, His163, Met165, Glu166, Gln189                           | 0.0145                 | Fluorogenic substrate enzyme inhibition assay | 4.2                    | RNA-qPCR quantitative assay in VeroE6 cells | >100 RNA-qPCR quantitative assay in VeroE6 cells | [32]      |
| YH-71                 | –            | Not described                                                            | 0.0321                 | –                               | 2.88                   | CPE reduction assay in VeroE6 cells | >100 Cytotoxicity assays in Vero E6 cells | [34]      |
| compound 4            | 7JT7/        | Gly143, His163, Glu166, Gln189                                         | 0.151                  | –                               | –                      | –                               | – –                  | [32]      |
|                      | 7JW8         |                                                                        |                        |                                 |                        |                                 |                      |                                 |           |
| 13b                   | 6Y2G         | Gly143, Phe140, Gly143, Ser144, Cys145, His163, Glu166                  | 0.67                   | FRET-based assay                 | 4–5                    | Antiviral activity assay in human Calu-3 lung cells | – –                  | [15]      |
| Boceprevir            | 7C6S         | His41, Gly143, Cys145, His164, Glu166                                  | 5.4/1.59               | FRET–based assay                 | 15.57                  | Plaque reduction assay          | – –                  | [23,35,36]|
| Narlaprevir           | 7JYC         | His41, Asn142, Gly143, His164                                          | 5.4/1.59               | FRET–based assay                 | 15.57                  | Plaque reduction assay          | – –                  | [23,35,36]|
| Telaprevir            | 7K0E/        | Gly143, Ser144, Glu166, Gln189                                         | 18                     | FRET–based assay                 | –                      | –                               | – –                  | [10,18]  |
|                      | 6XQS         |                                                                        |                        |                                 |                        |                                 |                      |                                 |           |
| ABT-957               | 7AEH         | Gly143, Cys145, His164, Glu166                                         | 3                      | Fluorescent peptide assay        | 10                     | CPE assay on HH7_mCherry cells  | >100 Cytotoxicity assay in HH7 cells | [39]      |
| Name                          | PDB ID | H-bonds | IC₅₀ (μM) | Calculation method | EC₅₀ (μM) | Calculation method | CC₅₀ (μM) | Calculation method | Reference |
|------------------------------|--------|---------|-----------|-------------------|-----------|-------------------|-----------|-------------------|-----------|
| Calpain inhibitor II         | –      | Not described | 0.97      | FRET-based assay  | 2.07/3.70 | CPE assay/ secondary viral yield reduction assay in Vero 76 cells | >100      | Cytotoxicity CPE assay on A549, MDCK, HCT-8 and Caco-2 cells | [38]      |
| Calpain inhibitor XII        | –      | His163, Glu166 | 0.45      | FRET-based assay  | 0.49/0.78 | CPE assay/ secondary viral yield reduction assay in Vero 76 cells | >100      | Cytotoxicity CPE assay on A549, MDCK, HCT-8 and Caco-2 cells | [38,56]   |
| Mg-132                       | 7BE7   | Not described | 0.36      | CPE assay in Vero E6 cells | –         | –                 | 2.9       | Vero E6 imaging assay | [40]      |
| Calpeptin                    | 7AKU   | His164, Glu166 | 0.072     | Fluorescent peptide assay | 100      | Antiviral activity assay in vero E6 cells | >100      | CCK8 assay in Vero E6 cells and HuH7 cells | [41]      |
| SDZ-224015                   | –      | Not described | 0.30      | Fluorescent peptide assay | 30.8/25.38 | Viral titer reduction assay on Vero E6/ HuH7 cells | >100      | Cytotoxicity assay in HuH7 cells | [42,43]   |
| Rupintrivir                  | 7LIU   | Not described | 0.68      | FRET-based assay  | 0.49/0.78 | Antiviral assay on Vero E6 cells | >300      | Cytotoxicity assay in Vero E6 cells (CCK8) | [45]      |
| Z-VAD(Ome)-FMK               | 7CUT   | Not described | 0.59      | FRET-based assay  | 1.88      | Antiviral assay on Vero E6 cells | >300      | Cytotoxicity assay in Vero E6 cells (CCK8) | [45]      |
| Z-DEVD-FMK                   | –      | Not described | 2.8       | FRET-based assay  | 0.87      | CPE assay on Vero E6 cells | >300      | Cytotoxicity assay in Vero E6 cells (CCK8) | [45]      |
| Z-IETD-FMK                   | –      | Not described | 1.61      | Fluorescent peptide assay | 0.64      | Antiviral assay on Vero E6 cells | >300      | Cytotoxicity assay in Vero E6 cells (CCK8) | [45]      |
| Tolperisone                  | 7ADW   | His163   | 19.17     | –                 | –         | Antiviral activity assay in vero E6 cells | >100      | CCK8 assay in Vero E6 cells | [41]      |
| Z-[1-(4-hydroxyphenyl)-ethylamino]methyl-tetralone (HEAT) | 6YNQ   | His163   | 24.05     | –                 | 0.59      | Fluorescent peptide assay | >100      | Cytotoxicity assays in Vero E6 cells | [34]      |
| Isofloxothepin               | 7AY7   | His163   | 4.8       | –                 | –         | –                 | 17        | –                 | [142]     |
| Triglycidyl isocyanurate     | 7AQj   | Gly143, Cln166, His163 | 30.02     | –                 | –         | –                 | >100      | –                 | [48]      |
| Quipazine maleate            | 7AHA   | Asn142, Gly143, Cys145 | 31.64     | –                 | –         | –                 | >100      | –                 | [49]      |
| MAC-5576                     | 7TIJ   | –       | 0.081     | Fluorescent peptide assay | –         | –                 | >100      | Cytotoxicity assays in Vero E6 cells | [34]      |
| Ebselen                      | 7BF8/  | His41, Cys145 | 0.67      | FRET-based cleavage assay | 4.67      | Plaque reduction assay | –         | –                 | [42]      |
| 7BAK                         | –      | Not described | 0.363     | FRET-based assay  | 4.5       | Antiviral activity assay on Vero E6 cells | –         | –                 | [47]      |
| MR6-7-2                      | –      | Not described | 0.345     | –                 | 3.74      | –                 | –         | –                 | [48]      |
| MR6-31-2                     | 7BAL   | His41, Cys145 | 0.824     | –                 | 1.78      | –                 | –         | –                 | [48]      |
| CarmOFur                     | 7BUY   | Gly143, Cys145 | 1.82      | FRET-based cleavage assay | 24.3      | qRT-PCR assay in Vero E6 cells | 133.4     | Cytotoxicity assays in Vero E6 cells | [48]      |
| Compound 7d                  | –      | Not described | 0.073     | FRET-based assay  | 15        | CPE assay on Vero E6 cells | –         | –                 | [49]      |
| Compound 1                   | –      | Not described | 0.25      | –                 | 2.8       | –                 | >100      | not specified | [50]      |
| x2754 (PG-COVA-34)          | 5RH7   | Not described | 0.35      | –                 | –         | –                 | –         | –                 | [42]      |
| x2705                       | 5RH7   | Not described | –         | –                 | –         | –                 | –         | –                 | [42]      |
| Nelfinavir                   | –      | Not described | 0.78      | FRET-based assay  | –         | –                 | –         | –                 | [42]      |
| Bedaquiline                  | –      | Thr26, Gly143, Glu166 | 18.7      | FRET-based assay  | –         | –                 | –         | –                 | [35]      |
| Manidipine                   | –      | Cys145   | 4.81      | FRET-based assay  | –         | –                 | –         | –                 | [35]      |
| Lercanidipine                | –      | Not described | 16.2      | –                 | –         | –                 | –         | –                 | [35]      |

**Non-covalent inhibitors**

| Perampanel                   | –      | Not described | 100-250   | FRET-based assay  | –         | –                 | –         | –                 | [35]      |
| Compound 2                   | –      | His163, Glu166, Cys145 | 6.4       | –                 | –         | –                 | –         | –                 | [53]      |
| Compound 3                   | –      | Thr26, His163, Glu166 | 4         | –                 | –         | –                 | –         | –                 | [53]      |
| Compound 4                   | 7L10   | Gly143, His163, Glu166 | 0.018     | FRET-based assay  | 11.3      | Viral plaque assay in Vero E6 cells | 1.7      | MTT dye assay in Vero E6 cells | [52]      |
| Compound 5                   | 7L11   | Gly143, His163, Met165 | 0.14      | FRET-based assay  | 1.5       | Plaque reduction assay | 22       | MTT dye assay in Vero E6 cells | [52]      |
| Compound 6                   | 7L14   | Not described | 0.17      | –                 | 0.98      | –                 | >100      | –                 | [55]      |
| ML 188                      | 7UDO   | Gly143, His163, Glu166 | 2.5       | FRET-based assay  | –         | –                 | –         | –                 | [54,56]   |
| ML300                       | 7UME   | Ser46, Cys145, His163, Glu166 | 4.99     | FRET-based assay  | 19.9      | CPE inhibition assay in Vero E6 cells | –         | –                 | [55]      |
| Compound 41 (CCF0038981)     | –      | Not described | 0.068     | –                 | 0.497     | –                 | >50       | CPE inhibition assay in Vero E6 cells | (continued on next page) |

(continued on next page)
| Name                | PDB ID | H-bonds               | IC<sub>50</sub> (μM) | Calculation method | EC<sub>50</sub> (μM) | Calculation method | CC<sub>50</sub> (μM) | Calculation method | Reference |
|---------------------|--------|-----------------------|-----------------------|---------------------|-----------------------|---------------------|---------------------|---------------------|-----------|
| 23R (Jun8-76-3A)   | 7KX5   | Gly143, His163        | 0.2                   | FRET–based assay    | 1.27                  | Antiviral activity assay in vero E6 cells | >100                | Cytopathy assays in vero E6 cells | [56]      |
| MUT056399          | 7AP6   | Phe140, His163        | –                     | –                   | 38.24                 | Antiviral activity assay in vero E6 cells | >100                | CCK8 in Vero E6 cells | [41]      |
| F01                | 7PS1   | Cys145, His163, Glu166| 54                    | FRET–based assay    | 1.2                  | Antiviral activity assay in vero-81 cells | >400                | Cytopathy assays in vero-81 cells | [57]      |
| Zinc acetate       | –      | –                     | 325.1                 | Enzyme inhibition   | 3.28                  | Antiviral activity assay in vero E6 cells | –                   | –                   | [58]      |
| Zinc glutamate     | –      | –                     | 405.3                 | assay               | No activity           | –                   | –                   | –                   | [58]      |
| Mucle-5948770040   | 7LTJ   | –                     | –                     | –                   | –                     | –                   | –                   | –                   | [58]      |
| x77                | 6 W63  | –                     | –                     | –                   | –                     | –                   | –                   | –                   | [58]      |
| x0104              | 5RZ7   | Not described         | –                     | –                   | –                     | –                   | –                   | –                   | [41]      |
| x0161              | 5R80   | Not described         | –                     | –                   | –                     | –                   | –                   | –                   | [41]      |
| x0397              | 5RGl   | Not described         | –                     | –                   | –                     | –                   | –                   | –                   | [41]      |
| Allosteric inhibitors |       |                       |                       |                     |                       |                     |                     |                     |           |
| Pelitinib          | 7AXM   | –                     | –                     | –                   | 1.25                  | Antiviral activity assay in vero E6 cells | 13.96               | CCK8 in Vero E6 cells | [41]      |
| AT7519             | 7ACA   | Glu110, Asp153        | –                     | –                   | 25.16                 | –                   | –                   | –                   | [41]      |
| Ilotroprid         | 7AQJ   | –                     | –                     | –                   | 46.86                 | –                   | >100                | –                   | [41]      |
| RS-102895          | 7ABU   | Asn142                | –                     | –                   | 19.8                  | –                   | 54.98               | –                   | [41]      |
| PDA-168568         | 7AMJ   | –                     | –                     | –                   | –                     | –                   | –                   | –                   | [41]      |
| Tofopaflozin       | 7APH   | –                     | –                     | –                   | –                     | –                   | –                   | –                   | [41]      |
| Inhibitors with unspecified binding mode |       |                       |                       |                     |                       |                     |                     |                     |           |
| Ciprofloxacin      | –      | Met49, Cys145, Met165, Glu166 | 5.13                 | 3CLPRO antiviral assay | 50.07 nM | qPCR viral load reduction assay on Vero cells | >16                 | MTT assay in Vero cells | [143]     |
| 7-(4-(N-substituted carbamoyl methyl) piperazin-1 yl)-chalcone | – | Gly143, Cys145 | 0.6 | 3.93 nM | – | Live virus-based microneutralization assay in Vero E6 and human A549/ACE2 cells | – | – | [41] |
| Pimozide           | –      | Not described         | 42                    | FRET–based assay    | –                     | –                   | –                   | –                   | [41]      |
| Ebastine           | –      | Not described         | 57                    | –                   | –                     | –                   | –                   | –                   | [41]      |
| Bepridil           | –      | Not described         | 72                    | 0.86/0.46           | –                     | –                   | –                   | –                   | [41]      |
| Seraconazole       | –      | Not described         | 76                    | –                   | –                     | –                   | –                   | –                   | [41]      |
| Rimonabant         | –      | Not described         | 85                    | –                   | –                     | –                   | –                   | –                   | [41]      |
| Oxiconazole        | –      | Not described         | 99                    | –                   | –                     | –                   | –                   | –                   | [41]      |
| Itraconazole       | –      | Not described         | 111                   | –                   | –                     | –                   | –                   | –                   | [41]      |
| Tipranavir         | –      | Not described         | 180                   | –                   | –                     | –                   | –                   | –                   | [41]      |
| Zopiclone          | –      | Not described         | 349                   | –                   | –                     | –                   | –                   | –                   | [41]      |
| Trihexyphenidyl    | –      | Not described         | 370                   | –                   | –                     | –                   | –                   | –                   | [41]      |
| Saquinavir         | –      | Not described         | 411                   | –                   | –                     | –                   | –                   | –                   | [41]      |
| Isavuconazole      | –      | Not described         | 438                   | –                   | –                     | –                   | –                   | –                   | [41]      |
| Lopinavir          | –      | Not described         | 486                   | FRET–based assay    | 12.01/ 7.79          | Viral titer reduction assay on Vero E6/ Huh7 cells | 80.82/ 64.43         | CCK8 assay in Vero E6/ Huh7 cells | [42,43] |
| Clemastine         | –      | Not described         | 497                   | FRET–based assay    | –                     | –                   | –                   | –                   | [42]      |
| Metoxine           | –      | Not described         | 635                   | –                   | –                     | –                   | –                   | –                   | [42]      |
| Duloxetine         | –      | Not described         | 3047                  | –                   | –                     | –                   | –                   | –                   | [42]      |
| Elfonidine         | –      | Not described         | 38.5                  | FRET–based assay    | –                     | –                   | –                   | –                   | [42]      |
| ALG-097111         | –      | Not described         | 0.007                 | Biochemical enzyme assay | 0.2           | Antiviral activity assay in A549/ACE2 | >100               | Cytopathy assays in A549/ACE2 | [59]     |
| Ritonavir          | –      | Not described         | –                     | –                   | 19.8/ 11.68           | Viral titer reduction assay on Vero E6/ Huh7 cells | 94.71/ 83.73         | CCK8 assay in Vero E6/ Huh7 cells | [43]     |
| Ag7404             | –      | Not described         | –                     | –                   | 195.8/ 92.55          | Viral titer reduction assay on Vero E6/ Huh7 cells | >400/ >400          | CCK8 assay in Vero E6/ Huh7 cells | [43]     |

1: Inhibition constant Kᵢ
2: Different sources provide different IC<sub>50</sub> values.
the place of the benzyl ring in the P3 position. The IC₅₀ values for the designed molecules were 2-fold lower than the parent compound GC376 (0.07 and 0.08 μM respectively, as opposed to 0.19 μM for GC376 in the same assay). Deuterated derivatives of GC376 have been tested in vitro and in vivo in mice and showed improved inhibitory activity compared to GC376.

Sodium (2S)-1-hydroxy-2-(S)-4-methyl-2-(((phenylmethoxyd2)carylcarbonyl)amino)pentanamido)-(3S)-2-oxopyrrolylidin-3-yl)propane-1-sulfonate, mentioned as compound 2 in the respective study, displayed a slightly enhanced IC₅₀ value, as low as 0.18 μM. Significantly higher inhibition of viral replication in Vero E6 and A549-AEC2 cells was observed, when the EC₅₀ values occurring from the respective antiviral assays were equal to 0.086 and 0.069 μM, respectively. Moreover, the cytotoxicity of the compound was low, as the CC₅₀ value occurring from cytotoxicity assays in Vero E6 and CRFK cells was >100 μM [25].

Yang et al. [26] designed a series of β-((S-2-oxopyrrolidin-3-yl)-alaninal (Opal)-based reversible covalent inhibitors, which include dipeptidyl and tripeptidyl compounds. Their design resembles inhibitor GC376. Both dipeptidyl compounds named MPI1 and MPI2 showed an IC₅₀ value approximately 100 nM, as opposed to 31 ± 4 nM for GC376, while the tripeptidyl structures yielded more encouraging results, with the most prominent compounds being MPI3, MPI4 and MPI5 with IC₅₀ values as low as 8.5 ± 1.5, 15 ± 5 and 33 ± 2 nM respectively. The highest IC₅₀, calculated via a fluorescence peptide assay, was 105 ± 22 nM for compound MPI3. However, a slow dissociation of the Mₚro in further in vitro investigations in Vero E6 cells. More specifically, compounds MPI5, MPI7 and MPI8 inhibited the protease more efficiently than GC376, completely blocking SARS-CoV-2 induced cytopathic effect (CPE) at concentrations of 5–2.5 μM, compared to 10 μM for GC376. When further tested in A549/AEC2 cells, which are considered more suitable to test the SARS-CoV-2 inhibitors than Vero E6 cells, as they can be used to more accurately resemble human respiratory tract infection, MPI5 and MPI8 completely hindered CPE at concentrations of 160–310 nM, considerably lower than inhibitor 11a, which has the same effect at concentration of 5 μM. Overall, observation of the interactions of the various designed inhibitors with the active site concludes that the leucine residues in the P2 position results in more favorable binding [26].

Two other covalent inhibitors are 11a and 11b, that both are covalently bound to the S-atom of Cys 145, with a 1.8 Å bond. The enzyme-inhibitor complex is further stabilized with a hydrogen bond between the oxygen of the aldehyde group of 11a and 11b and Cys145. Additionally, they both form one hydrogen bond with Phe140, His163 and His164 and three with Glu166. Inhibitor 11b contains a F-atom that forms an additional hydrogen bond with Gln189. The cyclohexyl group of 11a inserts the hydrophobic pocket that makes up the S2 subsite, showing hydrophobic interactions with residues His41, Met49, Tyr54, Asp187 and Arg188. The cyclohexyl group of 11a, forming hydrophobic interactions with residues His41, Met49, Met165, Val186, Asp187 and Arg188. An important role in the stabilization of the inhibitors is played by some water molecules, which form hydrogen bonds with both 11a/11b and the residues of the binding cleft. At a concentration of 1 μM, 11a and 11b exhibited 100% and 96% inhibitory activity, respectively. Moreover, the IC₅₀ values are promising, equaling 0.053 ± 0.005 μM for 11a and 0.040 ± 0.002 μM for 11b. Between the two inhibitors, results showed that 11a has a greater potential to act as an antiviral compound [9].

Xia et al. [27] have used superposition of the crystal structures of inhibitors GC376, telaprevir and boceprevir to design two novel hybrid inhibitors, which combine the chemical groups of their parent compounds that result in the most interactions and most favorable binding. The designed inhibitors are UWJ9-36-1, as a hybrid of GC376 and telaprevir, and UWJ9-36-3, as a hybrid of GC376 and boceprevir. Their inhibitory effect was evaluated via a fluorescence resonance energy transfer (FRET)-based enzyme inhibition assay, which resulted in IC₅₀ values of 0.051 and 0.054 μM, slightly higher that the respective value calculated for GC376 in the same assay (0.041 μM). To confirm the inhibitory activity of the compounds in a cellular environment, a Flip-GFP assay was used. The calculated IC₅₀ value for UWJ9-36-1 was 11.10 μM, while for UWJ9-36-3 was 3.40 μM. The latter exhibited greater inhibitory effect than GC376, for which IC₅₀ was calculated 4.83 μM in this assay. The synthesized compounds displayed inhibitory effect against the main proteases of other coronaviruses as well, including SARS-CoV, MERS-CoV, HCoV-OC43, HCoV-229E, HCoV-NL63 and HCoV-HKU1. Therefore, they reveal a path towards the development of broad-spectrum antivirals.

Another potent antiviral agent is MI-23, which has been designed based on telaprevir and exhibits IC₅₀ = 7.6 nM. It forms the characteristic 1.8 Å covalent bond with Cys145 and additionally hydrogen bonds with Phe140, Gly143, Cys145, His163, His164 and Glu166. The bicyclopentane moiety is located in the hydrophobic S2 subsite, having hydrophobic interactions with residues His41, Met49, Met165, Leu167, Pro168, Asp187, Arg188 and Gln189 [18].

PF-00835231 and its phosphate prodrug PF-07304814, is the first anti-Mₚro compound to proceed to clinical trials. PF-00835231 has been investigated in vitro and in vivo, providing indications of anti-SARS-CoV-2 activity, as well as synergistic effect with the FDA-approved drug remdesivir. A thermal-shift assay showed high affinity and specificity in the binding of PF-00835231 to Mₚro, while a FRET protease activity assay revealed inhibitory effect of the compound against various types of coronaviruses. Evaluation of the antiviral effect of the compounds in cells via the CPE assay yielded encouraging results, with EC₅₀ values equal to 0.23 μM in VeroE6–enACE2 cells and 0.76 μM in VeroE6–EGFP cells. This study was performed in the presence of the efflux transporter P-glycoprotein inhibitor, as the glycoprotein is expressed in Vero cells and PF-00835231 inhibits its action. Therefore, without the glycoprotein inhibitor, the concentration of the compound available to bind to Mₚro would be lower than the desired one [28]. A different study, however, points out that the effect of the glycoprotein is minimal in airway epithelial cells, which are mostly infected by SARS-CoV-2 [29]. The same study included a comparative assay performed on A549/AEC2 cells infected with two clades of SARS-CoV-2, where PF-00835231 showed better antiviral properties compared to RdRp inhibitor remdesivir. For clade A, the EC₅₀ value calculated at 24 h post infection was equal to 0.221 μM for PF-00835231, as opposed to 0.442 μM for remdesivir, while the respective values for clade B were 0.184 and 0.283 μM. In a different cell assay, comparing the viral inhibition of PF-00835231 with that of GC376, the former exhibited again more promising properties, with EC₅₀ values equal to 0.422 and 0.326 μM for clades A and B at 24 h post infection, compared to 0.632 and 0.529 μM for GC376 [29]. Lastly, it is worth mentioning that pharmacokinetic studies performed in rats and monkeys indicate short elimination-half life and limited oral bioavailability of the compound, suggesting that intravenous administration would be more efficient.

PF-07321332 is another highly potent Mₚro inhibitor, which has been designed for optimized oral bioavailability and has also been subjected to clinical trials. It covalently and reversibly binds to the catalytic cysteine through its nitrile warhead, also forming hydrogen bonds with residues His163, Glu166 and Gln189. Its inhibitory effect has been quantified through the CPE assay in Vero E6 cells, the nuclease reporter virus assay in A549/AEC2 cells and the viral titer reduction assay in differentiated normal human bronchial epithelial (dNHBE) cells. The assays resulted in EC₅₀ val-
Fig. 4. Binding mode and structure of covalent peptidomimetic inhibitors with a γ-lactam (colored red) or α-ketoamide (colored dark green) moiety, based on available co-crystallization PDB structures in the active site of SARS-CoV-2 Mpro. Catalytic residues are colored (His41: green, Cys145: yellow). Important residues for binding are shown in sticks and hydrogen bonds are depicted as yellow dashes. The PDB ID for each inhibitor is indicated in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
ues of 74.5, 77.9 and 61.8 nM respectively, while the compound cytotoxicity was considerably lower in Vero E6 compared to A549-ACE2 cells (CC50 > 100 μM and CC50 > 3 μM, respectively). A FRET-based assay allowed measurement of its inhibition constant against Mpro (Ki = 2.5 nM), while also providing indications of its inhibitory effect against the main proteases of other known alpha and beta-coronaviruses, including SARS-CoV-1, HKU1, OC43, MERS, 229E and NL63 [30,31]. A tetrapeptide inhibitor of SARS-CoV-1 has been the basis for the design of peptide-like derivatives with an aryl (and more specifically benzothiazolyl) ketone warhead through which they covalently bind to the sulfur atom of Cys145 of SARS-CoV-2 Mpro. Three such compounds with very similar structures (having a benzothiazole in the P1’ position, a pyrrolidine-2-one in the P1 position and an isobutyl group in the P2 position) have been investigated, namely SH-5, YH-53 and YH-71. The compounds inhibit both SARS-CoV-1 and SARS-CoV-2. The P1 group of the inhibitors interacts with residues His163 and Glu166 of SARS-CoV-2 Mpro through its carbonyl and amide groups, while the benzothiazole facilitates the formation of a hydrogen bond with His41. Particularly in the case of YH-53, its P2 amide forms a hydrogen bond with Gln189, resulting in a tighter binding. A fluorogenic substrate enzyme inhibition assay allowed the calculation of the KI values for the three compounds which were 14.5, 34.7 and 32.1 nM, respectively. In addition, the compounds hindered viral replication in Vero E6 cells at concentrations of 25, 10 and 25 μM while showing low cytotoxicity. The activity of YH-53 was reinforced in the presence of CP-100356, an MDR-1 efflux transporter inhibitor. Its favorable safety and toxicity profile also encourages its development as a candidate drug. However, it should be noted that its bioavailability in rats was estimated to be as low as 3.6%. Apart from that, in all the inhibitors of this category, the concentrations at which significant antiviral activity in cells was observed deviated from the respective concentrations for enzyme inhibition, indicating a difficulty in cell entry or maintenance of a high intracellular concentration of the molecules [32]. YH-53 emerged as the most potent among other known protease inhibitors in the study Hattori et al. [33] as well, under the name “compound 5 h”. The compound showed an EC50 equal to 4.2 ± 0.7 μM, while exhibiting low cytotoxicity with a CC50 value >100 μM. It is reported to form a reversible covalent bond with Cys145, via the same nucleophilic addition mechanism that other covalent inhibitors exhibit. More specifically, the sulfur atom of Cys145 attacks the carbonyl carbon next to the benzothiazole of compound 5 h. 5 h forms two hydrogen bonds with Glu166, and one with each of Cys145, His164 and Gln189. Also in this case, there are three water molecules that form hydrogen bonds with the inhibitor and the enzyme are mostly found in subsites S2 and S4, and more specifically with residues Met149, Met165, Asp187, Gln189, Thr190 and Gln192 [23]. Another molecule that displayed successful inhibition of Mpro is 4-[2-(Benzoylcarbonylamo)-3-tert-butoxy-butyrylamo]-4-methyl-pentanoylamino]-5-(2-oxo-pyrrolidin-3-yl)-pent-2-enoic acid ethyl ester (designated compound 4). It is a peptidomimetic molecule, which binds to Cys145 through Michael addition and blocks subsites S1 and S2. Its IC50 of 151 ± 15 nM was calculated in a fluorogenic peptide substrate enzymatic activity assay. The compound also hindered viral replication in Vero-E6 cells, as resulted from a cytotoxicity effect reduction assay from which an EC50 value of 2.88 ± 0.23 μM was derived [34]. 4.1.2. Peptidomimetic inhibitors with an α-ketoamide moiety Another structural characteristic observed in several inhibitors is the α-ketoamide warhead, whose one of the carbonyls forms a covalent bond with the catalytic cysteine. Alpha-ketoamide 13b is such a compound that also possesses a butyroloactam group in its P1 position. It has been found to covalently inhibit SARS-CoV-2 with IC50 = 0.67 ± 0.18 μM and EC50 = 4–5 μM. Its conformation in the binding site is further stabilized by six hydrogen bonds with residues His41, Phe140, Gly143, Ser144, Cys145, His163 and three hydrogen bonds with Glu166 [15]. Boceprevir was originally identified as a hepatitis C virus protease inhibitor and has been FDA-approved, therefore it has known toxicity and pharmacokinetic properties. It can effectively inhibit Mpro, as quantified by the IC50 value of 5.4 μM [35], while also limiting viral replication with an EC50 value of 15.57 μM. A different study on boceprevir reports a lower IC50 value of 1.59 μM, also calculated via a FRET-based assay [36]. The keto carbon of boceprevir is the atom that takes part in the covalent bond formation. There are also hydrogen bonds formed with residues His41, Gly143, Cys145, His164 and Glu166. In particular, Glu166 forms three hydrogen bonds with boceprevir. Hydrophobic interactions between the inhibitor and the enzyme are mostly found in subsites S2 and S4, and more specifically with residues Met149, Met165, Asp187, Gln189, Thr190 and Gln192 [23]. Naraneprevir is also a potent antiviral compound, with an IC50 value of 16.11 μM and EC50 value of 7.23 μM. According to literature, except for the covalent bond, it creates four hydrogen bonds with residues His41, Asn142, Gly143 and His164 and three hydrogen bonds with Glu166. It also interacts with residues Leu141, Ser144, Met165, Pro168 and Gln192 [37]. Binding to the active site of SARS-CoV-2 Mpro in a very similar way to naraneprevir and boceprevir, peptidomimetic compound telaprevir acts as an effective inhibitor, with an IC50 of 18 μM [10]. More specifically, apart from the covalent bond with Cys145, telaprevir forms direct hydrogen bonds with His41, Gly143, Ser144, His164, His166 (with which there are two interactions) and Gln189. There is also shown to be a water-mediated hydrogen bond with Glu192, as well as pi-pi interactions with residues Thr190 and Ala191 [18]. Calpain inhibitor XII is a cysteine protease inhibitor that exhibited an IC50 of 0.45 μM and an EC50 of 0.49 μM in a FRET-based and a CPE assay, respectively. A secondary viral yield reduction assay resulted in the calculation of an additional EC50 value, equal to 0.78 μM, while the compound also showed low cytotoxicity [38]. Another compound with an α-ketoamide group is a derivative of calpain 1 & 2, inhibitor ABT-957 [39]. It stands out due to its better pharmacokinetic properties and lower cytotoxicity compared to the other tested compounds, but it has a higher IC50 value of 3 μM, while other hits of the same study that will be mentioned below achieve inhibition at nanomolar levels. A summary of the binding mode and structure of the peptidomimetic inhibitors that include γ-lactam and/or a α-ketoamide moiety described above is presented in Fig. 4. 4.1.3. Other peptide-like inhibitors Apart from the previously mentioned calpain inhibitor XII, calpain inhibitor II also showed great potential in the inhibition of SARS-CoV-2 Mpro, inhibiting the protease with an IC50 value of 0.97 using a FRET-based assay. The evaluation of its antiviral activity yielded EC50 values of 2.07 and 3.70 in a CPE and a secondary viral yield reduction assay respectively, both in Vero76 cells. Moreover, it demonstrated low cytotoxicity (CC50 > 100 μM) [38]. Compound MG-132 is a reversible Mpro inhibitor (IC50 = 0.36 μM, CC50 = 2.9 μM), that also inhibits other cysteine proteases. Its relatively large size allows effective blocking of the subsites of the protein. Although it shows very effective inhibition of the protease, its high cytotoxicity poses a concern to its use a pharmaceutical compound [40]. Another peptidomimetic compound that has a comparable structure and binds in a similar manner to the binding site of Mpro is calpeptin. When in contact with the protease,
Cys145 attacks its aldehyde group to form a thiohemiacetal intermediate. The compound forms two hydrogen bonds, with residues His164 and Glu166. In addition, Van der Waals forces are developed between calpeptin and residues Phe140, Leu141 and Asn142. Due to these interactions, the inhibitor successfully blocks part of the active site, showing an EC<sub>50</sub> value of 72 nM and CC<sub>50</sub> value &gt;100 µM [41].

Emerging from the high throughput screening (HTS) of a library of compounds approved for investigation in humans, inhibitor SDZ-224015 is an irreversible covalent inhibitor that reacts with the catalytic cysteine. It includes three ester groups, one of which is cleaved in vivo by esterases, leading to the formation of a metabolite which, however, has lower potency against M<sup>pro</sup> inhibiting viral replication in HUH7_mCherry cells by 50% at 100 µM, as opposed to 10 µM by its prodrug. The HTS assay resulted in an IC<sub>50</sub> of 30 nM for SDZ-224015 [39].

Rupintrivir is a compound designed to inhibit 3C-proteases, having a lactone moiety in the P1 position that plays an important role in binding to the active site. Specifically against SARS-CoV-2 M<sup>pro</sup>, rupintrivir demonstrated low inhibition, with an IC<sub>50</sub> value of 68 µM [42]. A different study reports IC<sub>50</sub> values of 34.08 and 25.38 µM in viral titer reduction assays using Vero E6 and Huh7 cells, respectively, as well as a CC<sub>50</sub> value &gt;100 µM, as determined by the CCK8 assay in both cell types [43]. Lockbaum et al. [44] point out an interesting binding conformation of rupintrivir, which reveals an alternative mechanism of inhibition. Its fluorophenylalanine group, which normally occupies the S2 subsite in complexes of the molecule with other proteases, turns to the S1 subsite, acting as an obstacle between the two catalytic residues. However, other works characterize rupintrivir as a non-potent antiviral, due to its relatively high IC<sub>50</sub> and reported side effects in clinical trials [38]. An analogue of rupintrivir with enhanced oral bioavailability is AG7404. It inhibits viral replication in Vero E6 and Huh7 cells with IC<sub>50</sub> values of 195.8 and 92.55 µM respectively, while also showing low cytotoxicity in both cell types (CC<sub>50</sub> &gt; 400 µM) [43].

Caspase inhibitors also form another category of repurposed molecules that have been investigated and successfully inhibit M<sup>pro</sup>. The ones standing out possess a fluoromethylketone (FMK) moiety, which serves as a warhead for their covalent binding to the catalytic cysteine, as well as a non-bulky group in the P2 position. Three potent inhibitors identified include compounds Z-VAD (OMe)-FMK, Z-DEVD-FMK and Z-IEDT-FMK, whose activity against SARS-CoV-2 and cytotoxicity were evaluated through a FRET-based enzyme inhibition assay and antiviral assay on Vero cells. Z-VAD(OMe)-FMK showed an IC<sub>50</sub> value of 0.59 µM and an EC<sub>50</sub> of 1.88 µM, Z-DEVD-FMK demonstrated an IC<sub>50</sub> value of 2.80 µM and an EC<sub>50</sub> of 0.87 µM, while Z-IEDT-FMK IC<sub>50</sub> showed the IC<sub>50</sub> value of 1.61 µM and an EC<sub>50</sub> equal to 0.64 µM. All three compounds displayed low cytotoxicity (CC<sub>50</sub> &gt; 300 µM) [45]. A summary of the binding mode and structure of the peptidomimetic inhibitors described in this paragraph is presented in Fig. 5.

4.1.4. Small non-peptidic covalent inhibitors

The same study that reports calpeptin as an M<sup>pro</sup> inhibitor reported five other potent small compounds, which covalently bind to the active site of the protease [41]. These include tolperisone, 2-[β-(4-hydroxyphenyl)-ethylaminomethyl]-tetralone (HEAT), isofloxythepin, tricyglid isocyanurate and quipazine maleate, for which EC<sub>50</sub> values were 19.17, 24.05, 4.8, 30.02 and 31.64 µM. The CC<sub>50</sub> was estimated to be higher than 100 µM for all the compounds, with the exception of HEAT and isofloxythepin, for which it was 55.42 and 17.00 µM, respectively. It is also noteworthy that tricyglid isocyanurate shows indications of both covalent and non-covalent binding modes, inhibiting similar subsites of the active site (S1, S1' and S2).

Another non-peptidomimetic, small molecule with anti-SARS-CoV-2 M<sup>pro</sup> activity is MAC-5576, which covalently binds to the catalytic cysteine of the protease in a non time-dependent manner. It demonstrated a lower IC<sub>50</sub> value and equal to 81 ± 12 nM when compared to GC376 and compound 4, but did not show significant reduction of viral replication in Vero-E6 cells. The compounds showed no cytotoxicity in the tested concentrations (up to 100 µM). Overlay of the binding modes of the above mentioned inhibitors, as well as other previously mentioned inhibitors, such as GC376, 11a, 11b and N3, provides indications that the design of an effective inhibitor could initially focus in strong interactions with S1, S2 and/or S1’ subsites, and then be optimized to establish contacts with other parts of the active site [34].

Ebselen is an auspicious organoselenium drug molecule worth mentioning, as it inhibits the protease with an IC<sub>50</sub> of 0.67 µM and hinders viral replication with an EC<sub>50</sub> of 4.67 µM, while also exhibiting very low cytotoxicity. In the case of ebselen, covalent inhibition, which occurs by the creation of a bond between the selenium atom of the molecule and the thiol group of Cys145, is reinforced by its non-covalent interaction with the active site residues, which are however not described in detail [7,46]. Moreover, derivatives of ebselen have been investigated and displayed improved antiviral properties, both in terms of M<sup>pro</sup> inhibition, as well as in terms of limiting viral replication in cells [47]. More specifically, derivatives MR6-7-2 and MR6-18-4 inhibited the protease with IC<sub>50</sub> values of 0.363 and 0.345 µM, which are almost twice as low as ebselen, whereas derivative MR6-31-2 showed a remarkably higher antiviral effect in Vero cells, with an EC<sub>50</sub> of 1.78 µM.

Carfomur is an antineoplastic drug that has also been proved to inhibit M<sup>pro</sup>. Inhibitory effect and cytotoxicity have been tested on Vero E6 cells and resulted in an EC<sub>50</sub> value of 24.30 µM and a CC<sub>50</sub> value of 133.4 µM [48]. Unlike previous inhibitors that occupy multiple subsites of the protease, carfomur only binds to S2...
Fig. 6. Binding mode and structure of small covalent inhibitors with available co-crystallization PDB structures in the active site of SARS-CoV-2 Mpro. Catalytic residues are colored (His41: green, Cys145: yellow). Important residues for binding are shown in sticks and hydrogen bonds are depicted as yellow dashes. The PDB ID for each inhibitor is indicated in Table 1. *In the crystal structure of MR6-31–2 with the protease, only the selenium atom appears covalently bound to the active site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
subsite. The fact that this small compound is able to inhibit SARS-CoV-2 provides a good starting point from which more elaborate structures could be designed to inhibit the enzyme even more effectively. The mechanism through which the covalent bond is created is slightly different than the previously described cases, as the sulfur atom of Cys145 binds to the carbonyl group of the fatty acid tail of carmofur creating a 1.8 Å covalent bond. This reaction results in the release of the 5-fluorouracil moiety. The tail of carmofur inserts the S2 subsite and forms a hydrogen bond with each of Gly143 and Cys145. The conformation of the inhibitor in the active site is also affected by hydrophobic interactions with residues His41, Met49, Met165 and Asp187 [48].

Ghosh et al. [49] have evaluated 5-chloropyridin-3-yl ester derivatives with indole carboxylic acids for their inhibitory activity against Mpro. As deduced from the crystal structure of some representative derivatives in complex with the protease, the synthesized compounds covalently bind to the catalytic cysteine, forming a thioester bond through their indole carbonyl group. Among the derivatives investigated, the greatest potency in inhibiting Mpro was shown by 5-chloropyridin-3-yl 1-allyl-1H-indole-4-carboxylate (designated compound 7d), which includes an N-allyl substitution, with an IC50 of 0.073 µM as determined from a FRET-based enzyme inhibition assay. In a CPE assay on Vero E6 cells, the same compound exhibited an EC50 of 15 µM. In terms of the value of EC50, the most potent compound was 5-chloropyridin-3-yl 1H-indole-4-carboxylate (designated compound 1), for which EC50 was equal to 2.8 µM, more than five times lower than compound 7d, while it also displayed a low IC50 of 0.25 µM. Lastly, crystal structures that have been deposited to the PDB provide evidence of covalent inhibition of Mpro by various fragments. Two of them are PG-COV-34, or x2754, a small amide [50], and x2705, a more complex compound, for which the supporting paper has not been published yet. In both cases, there is no documented description of their interactions with the residues of the active site, but the crystal structure itself is an important indication. A summary of the binding mode and structure of the small non-peptidomimetic covalent inhibitors described in this paragraph is presented in Fig. 6.

4.2. Non-covalent inhibitors of Mpro

Known drugs that show inhibitory effect on Mpro include anti-tuberculosis drug bedaquiline (IC50 = 18.7 µM), HIV protease inhibitor neflinavir (IC50 = 234 µM), calcium channel blockers manidipine (IC50 = 4.81 µM), lercanidipine (IC50 = 16.2 µM) and efonidipine (IC50 = 38.5 µM) and glutamate receptor antagonist perampanel (IC50 = 100–250 µM) [51]. With the exception of perampanel, the lack of co-crystallization structure of the drugs in complex with the protease cannot confirm whether their binding is covalent or non-covalent. However, based on their structure, non-covalent inhibition would be expected. Perampanel, in particular, has been further investigated and served as a parent compound for the synthesis of optimized derivatives. Zhang et al. [52] used free-energy perturbation calculations and Vero E6 cell assays to investigate the inhibitory potential and antiviral properties of the different derivatives. Perampanel binds to the active site of Mpro with its pyridyl group occupying the S2 subsite, its phenyl group the S1 and its cyanophenyl group the S1’. Interactions were improved with reposition of the carbonyl group of perampanel from C2 to C6, as well as with an addition of a Cl atom in the benzene ring in the S2 subsite. This improvement was evident in compound 2 (2-(3-(3-Chlorophenyl)-2-oxo-2H-[1,3-bipyrindin]-5-yl)benzonitrile), compound 3 (5-(3-(3-Chlorophenyl)-2-oxo-2H-[1,3-bipyrindin]-5-yl)pyrimidine-2,4(1H,3H)-dione) and compound 4 (2-(3-(3,5-Dichlorophenyl)-2-oxo-2H-[1,3-bipyrindin]-5-yl)benzonitrile), which demonstrated IC50 values of 10.0, 6.4 and 4.0 µM, respectively. Further optimization of the interactions towards the S4 subsites yielded numerous effective inhibitors. Of them, the most effective inhibited the protease at nanomolar level concentrations. The lowest IC50 in this study was calculated for compound 21 (5-(3-(3-Chloro-5-((2-chlorobenzyl)oxy)phenyl)-2-oxo-2H-[1,3-bipyrindin]-5-yl)pyrimidine2,4(1H,3H)-dione) and was equal to 0.018 µM. The compound also showed antiviral activity through a lower-throughput viral plaque assay in Vero E6 cells, with an EC50 of 11.3 µM. Unfortunately, no activity was detected in a respective methylthiazolyl-diphenyl-tetrazolium bromide (MTT) assay and considerable cytotoxicity was observed (CC50 = 1.7 µM in Vero E6 cells). The two most promising compounds were compound 5 (2-(3-(3-Chloro-5-propoxyphenyl)-2-oxo-2H-[1,3-bipyrindin-5-yl]benzonitrile) and compound 26 (2-(3-(3-Chloro-5-((cyclopropylmethoxy)phenyl)-2-oxo-2H-[1,3-bipyrindin]-5-yl]benzonitrile). The difference in the structure of the two compounds is that the propyl group of compound 5 is replaced by a cyclopropyl group in compound 26. The calculated IC50 values for the two compounds were 0.140 µM and 0.170 µM respectively, indicating that the replacement of the propyl group by a cyclopropyl one leads to an increase of the IC50. The anti-SARS-CoV-2 activity of the two compounds is demonstrated by EC50 values of 1.5 and 0.98 µM, as measured with the plaque assay and 2.5 and 2.0 µM as calculated by the MTT assay. The cytotoxicity of compound 5 was significantly higher than compound 26, as indicated by the CC50 values measured in Vero E6 and normal human bronchial epithelial (NHBE) cells, which were as low as 22 and 20 µM, respectively, for compound 5 and higher than 100 µM in both cases for compound 26. Moreover, compound 5 provided evidence of synergy with remdesivir. In terms of interactions with the active site, compound 5 was shown to form three hydrogen bonds with active site residues Gly143, His163 and Met165, whereas the detailed interactions of compound 26 are not described [15,53].

A compound reported to inhibit SARS-CoV Mpro, ML 188, binds to the active site of SARS-CoV-2 Mpro as well, and inhibits its activity with an IC50 = 2.5 ± 0.3 µM. However, apart from pointing out the importance of the interaction with His41 for the inhibition, the interactions of the ligand with the active site are not described in detail [54]. Another molecule that inhibits both SARS-CoV and SARS-CoV-2 Mpro is ML300 and its derivatives have also demonstrated non-covalent inhibition. ML300 displayed an IC50 value of 4.99 µM, while the most eminent of its derivatives had a respective value of 0.106 µM. Moreover, its antiviral activity, as calculated by a CPE inhibition assay in Vero E6 cells, was quantified by an EC50 value of 19.9 µM. An eminente derivative is CFC0058981 (compound 41), which achieves inhibition at nanomolar concentration, with an IC50 of 68 nM, an EC50 of 497 nM and a CC50 of 50 µM [55]. Various non-covalent inhibitors of Mpro structurally related to ML 188 have been designed, synthesized and tested in vitro by Kita- murat et al. [56]. The IC50 values calculated for the originally designed compounds ranged from 0.28 to 20 µM. The ones that showed greater inhibition potency, while combining low cytotoxicity, were further evaluated in an antiviral immunofluorescence assay in Vero E6 cells and resulted in EC50 values ranging from 0.82 to 13.06 µM. Among these compounds, 23R (Jun8-76-3A), with an IC50 of 0.20 µM, an EC50 of 1.27 µM and low cytotoxicity, was selected for further investigation. A second antiviral assay in human lung epithelial Calu-3 cells displayed an EC50 of 3.03 µM. Moreover, insights into the binding mode of the inhibitor in the active site revealed its orientation in S1, S1’ and S2 subsites, as well as the formation of another subsite between S2 and S4 caused by the binding of the ligand that sheds light on an additional parameter that can be taken into consideration in drug design. It is also noteworthy that 23R exhibited selectivity towards coronavirus Mpro’s, when also tested among other viral proteases, as opposed to other inhibitors, such as GC376.
Fig. 7. Binding mode and structure of non-covalent inhibitors with available co-crystallization PDB structures in the active site of SARS-CoV-2 Mpro. Catalytic residues are colored (His41: green, Cys145: yellow). Important residues for binding are shown in sticks and hydrogen bonds are depicted as yellow dashes. The PDB ID for each inhibitor is indicated in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
MUT056399 is another compound that binds non-covalently to the active site, inhibiting it with an EC$_{50}$ of 38.24 μM. It also shows low cytotoxicity, as described by a CC$_{50}$ value > 100 μM. Its carboxamide group binds to the S1 subsite, forming hydrogen bonds with residues His163 and Phe140. The other end of the molecule, consisting of an ethyl-phenyl moiety, occupies S2 pocket [41].

Cantrelle et al. [57] have performed a fragment screening through which three binding hotspots of M pro and one particularly promising fragment have emerged. More specifically, two of the binding domains are located in the active site and the third one is found on the dimerization interface of the enzyme. The most eminent compound, named F01, was characterized as a reversible, non-covalent inhibitor, which inhibits the protease with an IC$_{50}$ of 54 μM as determined from an in vitro enzymatic assay. Moreover, the presence of F01 in SARS-CoV-2-infected Vero-81 cells resulted in the reduction of the concentration of the viral N-protein, described by an EC$_{50}$ value equal to 150 μM. The compound also exhibited low cytotoxicity (CC$_{50}$ > 400 μM). Therefore, F01 is an auspicious lead molecule, on which the design of optimized antiviral compounds can be based on.

Another interesting discovery is that of the inhibition of M pro by zinc ion (Zn$^{2+}$). Data indicated that ionic zinc reversibly forms a complex with the protease, aided by the presence of two crucial water molecules. An enzymatic activity assay testing zinc acetate allowed the determination of an IC$_{50}$ of 325.1 μM. Zinc glycinate and zinc gluconate also inhibited the protease, with IC$_{50}$ values of 279.4 and 405.3 μM, respectively. However, when the antiviral activity of the three zinc salts was tested in Vero E6 cells at their maximum non-toxic concentrations, only zinc acetate achieved 50% reduction of the viral titer, at a concentration of 3.227 μM. Additionally, the antiviral effect of Zn$^{2+}$ proved to be enhanced by the presence of quercetin. More specifically, quercetin at double the molar concentration of zinc acetate resulted in more than twice as high antiviral activity [58].

Also, among other inhibitors, available crystal structures for two compounds, Mcule-5948770040 and X77, prove their ability to non-covalently bind to the active site of the protease. The works framing the crystal structures though have not been published, therefore no additional information is available about them. However, the evident structural affinity between compounds X77 and ML188, which is also portrayed in their similar binding conformation in the active site of M pro, could be an indication of comparable antiviral properties. Regarding Mcule-5948770040, the respective co-crystallization structure shows that its pyrimidine group is stabilized in the S1 subsite, while the dichlorophenyl moiety is inserted into the S2 subsite and its sulfamoyl moiety blocking the S4 subsite. An interesting observation is related to the binding of compound x0397 (Z369936976), which interacts with the two catalytic residues changing their conformation. This alteration changes the shape of S1` subsite and consecutively the one of S1,
too. Therefore, this fragment blocks both sites, with its N-methyl group also providing the potential to block S2 and S3 subsites. Although there is a crystal structure that proves the binding of these inhibitors to the active site of M<sub>pro</sub>, there have not been in vitro experiments conducted yet to measure antiviral activity or cytotoxicity. A summary of the binding mode and structure of non-covalent inhibitors described in this paragraph is presented in Fig. 7.

### 4.3. Allosteric inhibitors

Günther et al. [41] discovered two regions outside the binding site of M<sub>pro</sub> that act as allosteric binding sites, as well as inhibitors that bind to these allosteric sites exhibiting remarkable antiviral activity. Residues Ile213, Leu253, Gln256, Val297 and Cys300 form a hydrophobic pocket that serves as the first allosteric binding site. Although pelitinib does not occupy the canonical active site of M<sub>pro</sub>, its ethyl ether group interacts with residues Tyr118 and Asn142, afecting the S1 pocket. The second allosteric inhibiting pocket is located in the cavity between domains I and II, and domain III. Inhibition affecting this site is connected to interactions of the inhibitor with residue Arg298, which plays a critical role in dimerization. Change in the conformation of Arg298 causes the alteration of the relative position of domains I/II and III and therefore destabilizes the oxyanion hole and the S1 subsite. Inhibitor AT7519 binds to this site forming Van der Waals contacts with residues Ile249 and Phe294 through its pyrazole ring. The carbonyl group interacts with Gln110 with a hydrogen bond and the piperidine group forms a hydrogen bond with Asp153. The reorientation of Asp153 is concomitant with a slight disposition of Tyr154 and its hydrogen-bonding to the inhibitor, as well as the interaction with Arg298, which is achieved through a salt bridge. The allosteric sites and the binding modes of the respective inhibitors are presented in Fig. 8.

### 4.4. Drug-like inhibitors with unspecified binding mode

Several drugs and drug-like molecules have been positively evaluated as promising SARS-CoV-2 inhibitors in vitro, but have not been co-crystallized with the protease or studied enough in order to provide a detailed description of the binding mode. Therefore, it is not confirmed whether the mode is covalent, non-covalent or allosteric. Such selective M<sub>pro</sub> inhibitor, whose activity has also been evaluated in vivo, is ALG-097111. The compound inhibits the protease with an IC<sub>50</sub> of 7 nM, while also exhibiting an EC<sub>50</sub> of 0.2 µM in A549-ACE2 cells and low cytotoxicity (CC<sub>50</sub> > 100 µM). When administrated to female SG hamsters, a day at a 200 mg/kg of dose in combination with ritonavir (50 mg/kg/dose) caused a 3.5log<sub>10</sub> reduction of viral titer compared to the control group, measured 2 days post infection. Thus, ALG-097111 may be another compound standing out as an interesting lead in drug development [59].

Vatansever et al. [42] conducted a screening of FDA-approved drugs for their potential to inhibit M<sub>pro</sub>, from which several mole-
molecules emerged. The lowest IC$_{50}$ value among the tested drugs in a FRET-based assay was calculated for pimozide, equal to 42 μM. Ebastine (IC$_{50}$ = 57 μM) was also a promising compound, structurally related to pimozide, as they both possess a diphenylmethyl moiety and the two aromatic rings which are inserted in S2 and S4 subsites. A similar geometry is observed in bepridil, due to the presence of a N-phenyl-N-benzylamine group, which also inhibits the protease with an IC$_{50}$ of 72 μM. The three drugs were also tested in Vero E6 and human A549/ACE2 cells via a live virus-based microneutralization assay. Only bepridil hindered CPE, with an EC$_{50}$ of 0.86 and 0.46 in the two cell lines, respectively. Other small drug molecules with inhibitory effect against M$^{pro}$ are sertaconazole (IC$_{50}$ = 76 μM), rimonabant (IC$_{50}$ = 85 μM), oxiconazole (IC$_{50}$ = 99 μM), itraconazole (IC$_{50}$ = 111 μM), azacloglute (IC$_{50}$ = 180 μM), zopiclone (IC$_{50}$ = 349 μM), trihexyphenidyl (IC$_{50}$ = 370 μM), saquinavir (IC$_{50}$ = 411 μM), isavuconazole (IC$_{50}$ = 438 μM), lopinavir (IC$_{50}$ = 486 μM), clemastine (IC$_{50}$ = 497 μM), metaxalone (IC$_{50}$ = 635 μM) and duloxetine (IC$_{50}$ = 3047 μM). In another study, much lower IC$_{50}$ values were calculated for lopinavir in Vero E6 and Huh7 cells (12.01 and 7.79 μM, respectively). Ritonavir was also tested and resulted in respective IC$_{50}$ values of 19.88 and 11.68 μM, while also showing slightly lower cytotoxicity. A time-of-drug-addition assay for the two compounds located their activity at the post-entry stage of infection. However, a low free plasma concentration compared to the IC$_{50}$ values, as designated from an In Vitro to In Vivo Extrapolation analysis, is discouraging for the further investigation of the compounds as antiviral agents [43].

Additional compounds with an inhibitory effect, which could not however be reliably quantified due to incomplete inhibition at the maximum concentration tested in the assay, include dopamine D1 receptor antagonist pericazine, histamine H1-receptors antagonist azelastine, prostaglandin synthesis inhibitor cinnamom, topoisomerase II inhibitor idarubicin and anti-bacterial drugs clofamizine and talampicillin [35,42].

5. Drugs with M$^{pro}$ inhibitory effect that have proceeded to in vivo or clinical trials

Several repurposed drugs or newly designed compounds have been selected to be further evaluated in vivo or clinically. Among them, covalent M$^{pro}$ inhibitor PF-07321332 (Nirmatrelvir) has exhibited high bioavailability and antiviral activity when tested in mice and humans. In the form of the oral antiviral drug Paxlovid™ (Nirmatrelvir/ritonavir tablets) developed by Pfizer, it received Emergency Use Authorization by FDA [62]. Moreover,
Table 3
Natural sources with inhibitory activity against SARS-CoV-2 Mpro demonstrated in silico and in vitro studies.

| Compounds         | Plant source                                               | IC₅₀     | Calculation method                  | Binding energy (kcal/mol) | Software     | PDB ID    | H-bonds              | Reference |
|-------------------|------------------------------------------------------------|----------|------------------------------------|---------------------------|--------------|-----------|----------------------|-----------|
| Myricetin         | Polygoni avicularis, Moringa oleifera, Syzygium aromaticum | 3.68 μM  | FRET-based assay                   | −8.47                     | Glide XP     | 6LZE      | Phe140, Glu166, Asp187 | [106]     |
|                   |                                                            | 0.22 μM  | FRET-based assay                   | −                      |              |           |                      |           |
|                   |                                                            | 2.86 μM  | Colorimetric substrate enzyme inhibition assay | −                      |              |           |                      |           |
|                   |                                                            | 0.63 μM  | FRET-based assay                   | −                      |              |           |                      |           |
| Dihydromyricetin  | Amelopsis japonica                                         | 1.14 μM  | FRET-based assay                   | −                      |              |           |                      |           |
|                   |                                                            | 1.20 μM  | Colorimetric substrate enzyme inhibition assay | −                      |              |           |                      |           |
| Kaempferol        |                                                            | 34.5 μM  | CPE inhibition assay               | −6.4                     | AutoDock Vina|           | Not mentioned       | [109]     |
| Quercetin         | Azadirachta indica, Mangifera indica, Moringa oleifera, Citrus limon, Alum cepa, Alum sativum, Trigonella fornum-graecum, Mentha piperita | 7.40μM  | FRET-based assay                   | −7.2/-7.5                | AutoDock Vina| 6Y2E/6Y2F | Phe140, Leu141, Asn142, His163, Glu166, Arg188, Thr24, Thr25, Thr26, Cys145, Gly143, Asn142, Ser144, Met165 Leu141, Ser144, His163, Glu189 | [111]     |
| Rutin             | Pimenta dioica, Manilkara hexandra, Calendula officinalis  | 31.0 μg/mL | CPE inhibition assay in Vero E6 cells | −9.19                   | AutoDock Vina| 6LU7      |                       |           |
| Quercetagetin     | Eriocaulon buerferianum, Citrus unshiu                    | 1.24 μM  | Colorimetric substrate enzyme inhibition assay | −                      | AutoDock Vina|           |                      |           |
| Compounds                      | Plant source                          | IC<sub>50</sub>  | Calculation method          | Binding energy (kcal/mol) | Software | PDB ID | H-bonds                  | Reference |
|-------------------------------|---------------------------------------|------------------|-----------------------------|---------------------------|----------|--------|---------------------------|-----------|
| Gallic acid                   | *Pimenta doica*                       | 108 µg/mL        | CPE inhibition assay in Vero E6 cells | –4.52                     | MOE 2019.012 | 6LU7   | Phe140, Gly143, Glu166, Thr190 | [116]     |
| Epigallocatechin-3-O-gallate  | Green tea, muscadine grape, cacao     | 7.51 µM          | Fluorescent substrate enzyme assay | –8.7                      | Autodock Vina | 6LU7   | Not described             | [119]     |
|                              |                                       | 7.58 µg/mL       | FRET-based assay            | –                         | –         | –      | Glu166                    | [120]     |
|                              |                                       | –                | –                           | –7.8                      | Autodock Vina | 6LU7   | Asn142, Met165, Thr190    | [146]     |
|                              |                                       | –                | –                           | –7.6                      | Autodock Vina | 6LU7   | Thr26, His41, Gly143, Ser144, Cys145, Glu166, Glu189 | [147]     |
| Gallic acid                   | Green tea, muscadine grape, cacao     | 6.38 µM          | Fluorescent substrate enzyme assay | –8.7                      | Autodock Vina | 6LU7   | Leu141, His163, Arg188, Glu189, Thr190 | [148]     |
| Epicatechin-3-O-gallate       | Green tea, muscadine grape, cacao     | 5.21 µM          | Fluorescent substrate enzyme assay | –8.7                      | Autodock Vina | 6LU7   | Thr26, Met49, Glu166, Glu189, Thr190 | [121]     |
| Catechin-3-O-gallate          |                                       | 2.98 µM          | Fluorescent substrate enzyme assay | –8.3                      | Autodock Vina | 6LU7   | Thr26, Met49, Glu166, Glu189, Thr190 | [121]     |
| Theaflavin                    | black tea                             | 8.44 µg/mL       | FRET-based assay            | –                         | –         | –      | Tyr54, Thr190             | [120]     |
| Naringenin                    | Not mentioned                         | 92.0 µM          | FRET based assay            | –7.83                     | Glide 6 W63 | 6LU7   | Thr26, Met49, Glu166, Glu189, Thr190 | [121]     |
| Apigenin-7-O-glucoside        |                                       | 74.0 µM          | –                            | –7.56                     | –         | –      | Thr26, Met49, Glu166, Glu189, Thr190 | [121]     |
| Sennoside B                   |                                       | 104 µM           | –                            | –9.01                     | –         | –      | Thr190, Glu166, Asn142, Cys44 | [121]     |
| 2,3,4,5,6-pentahydroxybenzophenone |                                      | 102 µM           | –                            | –8.34                     | –         | –      | Arg188, Tyr54, Asn142, His164, Met165, Arg188 | [121]     |
| Curcumin                      | *Curcuma longa*                       | 75.0 µg/mL<sup>6</sup> | FRET-based assay          | –                         | –         | –      | Arg188, Tyr54, Asn142, His164, Met165, Arg188 | [122]     |
|                              |                                       | –                | –                           | –7.1                      | Autodock Vina | 6LU7   | Gly143, Ser144             | [130]     |
|                              |                                       | –                | –                           | –8.09                     | Glide 6 W63 | 6LU7   | Not described             | [124]     |
|                              |                                       | –                | –                           | –7.028<sup>3</sup>        | CoVDock 6LU7 | 6LU7   | Thr26, Gly143             | [113]     |
| Chlorogenic acid              | *Pimenta doicam*                      | 360 µg/mL        | CPE inhibition assay in Vero E6 cells | –7.18                    | MOE 2019.012 | 6LU7   | Not described             | [116]     |
|                              | *Moringa oleifera*                    | 39.5 µM          | FRET-based assay            | –                         | –         | –      | Not described             | [101]     |
|                              |                                       | –                | –                           | –7.2                      | Autodock Vina | 6LU7   | Cys145, His163, Arg188, Thr190, Glu192 | [107]     |
| Taicalin                      | *Scutellaria baicalensis*             | 6.41 µM          | FRET-based assay            | –                         | –         | –      | Not described             | [103]     |
|                              |                                       | 83.4 µM          | FRET-based assay            | –                         | –         | –      | Not described             | [105]     |
| Compounds          | Plant source          | IC₅₀ | Calculation method | Binding energy (kcal/mol) | Software | PDB ID | H-bonds                  | Reference   |
|--------------------|-----------------------|------|--------------------|---------------------------|----------|--------|--------------------------|-------------|
|                    |                        |      |                    |                            |          |        |                          |             |
| Baicalein          | Scutellaria baicalensis | 0.94 | µM                 | FRET-based assay          | –        | 6LU7   | Leu141, Gly143, Ser144, His163, Glu166 | [103]       |
|                    |                       | 0.39 | µM                 | Colorimetric substrate enzyme inhibition assay | –        | 6LU7   | Not described             | [105]       |
| Scutellarein       | Scutellaria genus, Erigerontis herba | 3.02 | µM                 | FRET-based assay          | –        | 6LU7   | Not described             | [103]       |
|                    |                       | 5.80 | µM                 | Colorimetric substrate enzyme inhibition assay | –        | 6LU7   | Not described             | [105]       |
| Forsythoside A     | Shuanghuanglian preparation | 3.18 | µM                 | FRET-based assay          | –        | 6LU7   | Not described             | [103]       |
| Forsythoside B     |                       | 2.88 | µM                 | FRET-based assay          | –        | 6LU7   | Not described             | [103]       |
| Forsythoside E     |                       | 6.68 | µM                 | Fluorescent substrate assay | –        | Autodock Vina | Not described             | [128]       |
| Forsythoside H     |                       | 10.2 | µM                 | Fluorescent substrate assay | –        | Autodock Vina | Not described             | [128]       |
| Forsythoside I     |                       | 5.47 | µM                 | Fluorescent substrate assay | –        | Autodock Vina | Not described             | [128]       |
| Isoforsythoside    |                       | 5.85 | µM                 | Fluorescent substrate assay | –        | Autodock Vina | Not described             | [128]       |
| Betulinic acid     | Olea europaea         | 14.6 | µM                 | Fluorescent substrate assay | –        | 6LU7   | Not described             | [128]       |
| Betulin            |                       | 89.7 | µM                 | Fluorescent substrate assay | –        | 6LU7   | Not described             | [128]       |
|                    |                       | 12.6 | µM                 | Fluorescent substrate assay | –        | 6LU7   | Not described             | [128]       |
| Ursolic acid       |                       | –    | –                  | –                         | –        | 6LU7   | Not described             | [128]       |
|                    |                       | –    | –                  | –                         | –        | 6LU7   | Not described             | [128]       |
| Maslinic acid      | Olea europaea         | 3.22 | µM                 | Fluorescent substrate assay | –        | 6LU7   | Not described             | [128]       |
| Glycyrrhizin       | Not mentioned         | 0.44 | mg/mL              | Antiviral activity assay on Vero E6 cells | –        | 6LU7   | Not described             | [132]       |
|                    |                       | –    | –                  | –                         | –        | 6LU7   | Not described             | [132]       |
|                    |                       | –    | –                  | –                         | –        | 6LU7   | Not described             | [132]       |
|                    |                       | –    | –                  | –                         | –        | 6LU7   | Not described             | [132]       |
| Vanicoside A       | Reynoutria japonica, Reynoutria sachalinensis | 23.1 | µM                 | Fluorescence substrate enzyme assay | 115.78  | GOLD 5.7.2 | Not described             | [135]       |
| Vanicoside B       | Reynoutria japonica, Reynoutria sachalinensis | 43.6 | µM                 | Fluorescence substrate enzyme assay | 129.7   | GOLD 5.7.2 | Not described             | [135]       |
| Acteoside          | Olea europaea, Verbascum phlomoides | 43.0 | µM                 | FRET based assay          | –        | 6 LU6 | Not described             | [121]       |

(continued on next page)
PF-07312332 has proven to be effective against emerging SARS-CoV-2 variants, including Lambda (B.1.1.318), B.1.2, Beta (B.1.351), Omicron (B.1.529), Zeta (P.2) and Delta (B.1.617.2), highlighting its universal potency for battling SARS-CoV-2 throughout various stages of the pandemic [63,64]. PF-07304814 (Lufotrelvir), which is the produg of PF-00835231, is another covalent inhibitor which has been proposed for intravenous administration and has completed its Phase 1 clinical trial in humans, after showing reduction of viral titer in SARS-CoV-2 infected mice [65,66]. PF-00835231 has exhibited in vitro inhibitory effect against SARS-CoV-2 Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.529) variants as well [67].

Another drug being evaluated in phase I clinical trials is PBI-0451, a covalent, reversible and orally administered Mpro inhibitor developed by Pardes Biosciences, Inc. There is a lack of published in vitro or in vivo data, but the company reports efficiency of the drug against SARS-CoV-2 and its variants, while FDA recently cleared the Investigational New Drug (IND) application submitted for the compound [68,69]. The case is similar for oral protease inhibitor EDP-235, developed by the company Enanta Pharmaceuticals, which has also been reported to have promising antiviral and pharmacokinetic properties. EDP-235 is in Phase 1 of clinical trials [70]. Data emerging from in vitro biochemical and antiviral assays in human airway epithelial cells include an IC50 value of 5.8 nm and an EC50 value of 33 nM respectively [71].

S-217622 is a non-peptidic, non-covalent Mpro inhibitor effective at nanomolar levels (IC50 = 13 nM, as calculated from an enzymatic inhibition assay). Its efficiency in restricting viral replication in infected mice, as well as good pharmacokinetic properties and oral bioavailability have led to its further investigation in clinical trials. Currently, it is in the phase 2b/3, while its efficiency has been confirmed in the phase 2a [72,73]. Other protease inhibitors in phase 2 of clinical trials include atazanavir, which has already exhibited an EC50 of 0.49 μM in an antiviral assay in Calu-3 cells, as well as a 30% increase of survival in infected mice [74,75], ebselen (SPI-1005) [76] and lopinavir/ritonavir [77]. The latter, however, has been reported to have no significant efficacy against SARS-CoV-2 in both in vitro or clinical studies [78,79]. It is worth mentioning that atazanavir in particular is potent against SARS-CoV-2B.1 strains as well as the Gamma variant, as determined from in vitro studies in Calu-3 cells and in vivo in mice [74], while lopinavir has shown very similar binding affinity to the Mpro of the Omicron variant as opposed to that of the wildtype in silico [80].

Danoprevir is a repurposed non-covalent hepatitis C virus protease inhibitor that has been positively evaluated for its antiviral effect when administered orally, in combination with ritonavir, to COVID-19 patients and has completed phase 4 of clinical studies [81,82]. Its anti-SARS-CoV-2 activity has been confirmed in vitro, with an EC50 of 87 μM calculated from an antiviral assay in vero E6 cells [83]. In addition, previously described inhibitors 13b and GC376 are in preclinical stage, with 13b having exhibited encouraging pharmacokinetic properties in mice [15] and GC376 having resulted in limitation of viral load and mitigation of symptoms in infected K18-hACE2 transgenic mice, such as tissue lesions and inflammation [84]. 13b has also been evaluated via molecular docking for its efficacy against the Omicron variant and has exhibited slightly higher binding affinity compared to the wildtype [80]. The available data regarding the aforementioned inhibitors is summed up in Table 2 while their structures, if available, are presented in Fig. 9.

There are several drugs with promising activity against SARS-CoV-2 for which the evidence to support whether their antiviral activity is attributed to inhibition of Mpro is not conclusive. This may be due to the potential drug implication with more than one mechanisms related to the viral life cycle. Being part of the category of HIV protease inhibitors (which also includes previously mentioned drugs atazanavir and lopinavir/ritonavir), darunavir is
a compound that is presently in phase 3 of clinical trials, where it is being evaluated in combination with cobicistat [85]. Both compounds have indicated considerable binding affinity to M\textsuperscript{pro} in in silico simulations [86]. However, when tested in a cellular assay, an IC\textsubscript{50} of 36.1 μM was calculated for darunavir (as opposed to 10.9 μM for lopinavir/ritonavir and 60.7 μM for atazanavir in the same assay), but no inhibitory effect was observed at 100 μM in an enzyme inhibition assay [87]. As for cobicistat, in vitro results from an enzyme inhibition assay report an IC\textsubscript{50} of 6.7 μM [88], while another study refutes these results, reporting no inhibition of M\textsuperscript{pro} [89]. Therefore, the antiviral activity of the two drugs cannot be certainly attributed to the inhibition of the main protease.

Celecoxib is a drug currently in phase 2 of clinical trials [90] that is mainly reported as a cyclooxygenase 2 inhibitor [91]. There are indications of inhibitory activity against M\textsuperscript{pro}, resulting in 11.90% inhibition at 50 μM [92]. Dexamethasone is a drug with significant anti-inflammatory properties, that is now in phase 4 clinical trials against COVID-19 [93]. It is mainly reported to have high binding affinity to the glucocorticoid receptor and various cytokines, such as interleukin-6, but it has also emerged as a potential M\textsuperscript{pro} inhibitor from in silico studies [94,95]. Likewise, doxycycline is a compound highlighted for its anti-inflammatory properties that is in phase 4 of clinical trials against COVID-19 [96]. It shows anti-SARS-CoV-2 activity in vitro [97], but there have only been in silico studies supporting the hypothesis that it can inhibit M\textsuperscript{pro} [98]. All-trans retinoic acid is a compound that is being evaluated in Phase 2 clinical trials as a chemopreventive agent, with no reference being made to its potential M\textsuperscript{pro} inhibitory activity in vivo [99]. However, such activity is demonstrated in an IC\textsubscript{50} value of 24.7 μM calculated through an in vitro enzyme inhibition assay, while the compound also shows antiviral activity in Calu-3 cells against SARS-CoV-2 and its alpha, beta, gamma and delta mutants, with respective IC\textsubscript{50} values as low as 0.66, 0.97, 0.87 and 0.79 μM, as determined from an RT-PCR assay [100]. It is interesting to point out that over the course of the COVID-19 pandemic, various mutations were observed in the genes encoding major viral proteins. Among them, the spike protein is the most vulnerable. Interestingly, only few mutations have been reported for M\textsuperscript{pro} of the SARS-CoV-2 variants. For example, in the case of the omicron variant, only one mutation was observed for M\textsuperscript{pro}, as opposed to 36 for the spike protein [80]. Another study investigating frequent SARS-CoV-2 M\textsuperscript{pro} mutants reports six dominant mutations observed in the Lambda, B.1.1.318, B.1.2, Beta, Omicron and Zeta variants [63]. However, as mentioned above, several protease inhibitors remain effective against the main proteases of SARS-CoV-2 variants as well, reinforcing the reliability of M\textsuperscript{pro} as an antiviral target. Moreover, research has proceeded to the study of mutations and their impact on the structure and function of the protease, providing useful insights for the development of mutation-resistant inhibitors. A pathway towards such development may be the identification of residues playing an important role in the formation of the active site and the dimeric form of M\textsuperscript{pro} as mutation coldspots [101,102].

6. Natural compounds as inhibitors of M\textsuperscript{pro}

Apart from drug discovery and repurposing, research has been orientated towards phytochemicals in search for ways to restrain the effect that COVID-19 has on public health. Such strategy may reinforce the action of antiviral drugs and vaccines, which are much more time-consuming to be developed. Natural compounds found in extracts of plants, may be employed, as a tool for boosting immunity and aid protection against infection. Moreover, knowledge on the beneficial action of bioactive phytochemicals, may enhance preparedness for future viral outbreaks. Such phytochemicals may be used for the development of functional food supple-

ments or other functional aids. An overview of reported natural compounds that have demonstrated inhibitory activity against M\textsuperscript{pro} based on in silico and in vitro methods is presented in Table 3.

Myricetin is a naturally occurring flavonoid that has been identified as an M\textsuperscript{pro} inhibitor, and one of the few natural compounds that has been co-crystallized in complex with the protease. The respective structure has been deposited in the PDB under the ID 7B3E. Among the numerous natural myricetin sources, some reported in literature include plants Polygony avicularis, Moringa oleifera and Syzygium aromaticum. A FRET-based enzyme assay demonstrated IC\textsubscript{50} value of 0.63 for myricetin, while further evaluation of its antiviral effect in Vero E6 cells led to the calculation of EC\textsubscript{50} value of 8.00 μM. It is interesting that 2,4-dihydromyricetin, the respective flavanone of myricetin, also abundant in natural sources, was tested in the same assays and exhibited higher M\textsuperscript{pro} inhibitory effect but slightly lower antiviral efficacy overall, with IC\textsubscript{50} and EC\textsubscript{50} values of 1.14 and 13.56 μM, respectively [103]. The binding mode of myricetin, as determined through the crystal structure of its complex with M\textsuperscript{pro}, reveals the crucial role of its pyrogallol group. The group has the role of an electrophilic core and forms a covalent bond with the nucleophilic sulfur atom of the catalytic cysteine, while its hydroxyl moieties form hydrogen bonds with residues Thr26, Gly143, Ser144 and Cys145 [104]. The interactions that the pyrogallol group forms with the active site residues make it a promising potential potential weapon for the development of optimized inhibitors. For example, a methyl derivative of myricetin, substituted at its 7-OH, displayed improved properties with an IC\textsubscript{50} of 0.30 μM and an EC\textsubscript{50} of 12.59 μM. Bulkier substitutions in the same positions inhibited the protease, but in higher concentrations. Interestingly, comparison of the binding modes of baikalin and myricetin, both of which have a similar backbone that includes a pyrogallol moiety, highlights major differences, such as the fact that baikalin binds non-covalently to the active site, as opposed to myricetin. In both cases, however, pyrogallol participates in interactions that considerably contribute to the stabilization of the molecule in the active site [103].

Myricetin has been widely investigated in literature, leading to the reporting of various IC\textsubscript{50} values. Kuzikov et al. [40] calculated an IC\textsubscript{50} as low as 0.22 μM, through a FRET-based cleavage assay, while Liu et al. [105] mention an IC\textsubscript{50} of 2.86 μM, through a colorimetric substrate enzyme inhibition assay. A different study reports an IC\textsubscript{50} of 3.68 μM and conducted molecular docking simulations to investigate the binding of myricetin to M\textsuperscript{pro} (PDB ID: 6LZE) [106]. The calculated a binding energy was equal to −8.47 kcal/mol using the extra precision protocol of Glide software [106]. The simulation also predicted the formation of hydrogen bonds with residues Phε140, Glu166 and Asp187 and interaction with His41. Molecular docking simulation with a different software (AutoDock Vina) and on a different protease structure (PDB ID: 6LU7) resulted in binding energy of −7.7 kcal/mol [107].

Myricetin glycosides also seem potent. In the case of myricitrin, a 3-rhamnopyranoside of myricetin, the inhibitory effect was found weaker compared to myricetin, as the compound results in a 30.8% inhibition at a concentration of 50 μM [105], while another study reports a binding energy of −7.2 kcal/mol to the active site of M\textsuperscript{pro} [108]. Another derivative, myricetin-3-O-rutinoside, detected in Limoniastrum Guyonianum, has not been tested in vitro, but displayed good binding affinity to the protease in a molecular docking simulation, with a high binding energy of −9.0 kcal/mol.

Another representative of the group of flavonoids with promising indications of antiviral activity is kaempferol. A CPE inhibition assay in Vero E6 cells led to the calculation of an IC\textsubscript{50} value of 34.5 μM, while molecular docking studies have revealed multiple possible hydrogen bonds that can be formed between the com-
Fig. 10. Binding mode and structure of flavonoids, flavanones and derivatives with in vitro demonstrated inhibitory activity in the active site of SARS-CoV-2 Mpro. Catalytic residues are colored (H41: Green, C145: yellow), residues participating in hydrogen bonds are shown in sticks and hydrogen bonds are depicted as yellow dashes.1: Procyanidin B2 3,3′-di-O-gallate. The receptor-ligand complex was produced by docking simulations using the software YARASA Structure, replicating the binding mode represented in the relevant publication (Available in Table 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
pound and important active site residues, such as Phe140, Leu141, His163 and Glu166 [109,110].

Quercetin is another flavonoid with confirmed in vitro anti-SARS-CoV-2 Mpro activity. A FRET-based assay has shown effective inhibition, with the activity of the enzyme dropping below 10% at a quercetin concentration of 125 μM. Its inhibitory effect is described by an estimated intrinsic inhibition constant Ki = 7.4 μM [111]. In silico molecular docking and modelling of its binding to the active site reveal binding energies ranging from −8.5 to −7.2 kcal/mol and key hydrogen bond interactions which differ from study to study and include residues Leu141, Asn142, Ser 144, His163, Glu166 and Gln189 [58,107,112,113]. Numerous quercetin derivatives have also been investigated. More specifically, quercetin-3-0-glucoside (also known as isouqueretin) and quercetin rhamnoside (quercitrin) exhibited a better binding energy than remdesivir in the same molecular docking study (-8.2 and −8.6 kcal/mol respectively, as opposed to −7.9 kcal/mol for remdesivir). Their hydrogen bond interactions include residues similar to these of quercetin [114]. Rutin is also a derivative of quercetin (quercetin-3-O-rutinoside) with high anti-SARS-CoV-2 potency proven in vitro. Its inhibitory potential has been evaluated in a CPE reduction assay in Vero E6 cells, from which an IC50 value of 31 μg/mL was calculated. Cytotoxicity was low, as depicted in the CC50 value and equal to 8017 μg/mL. Molecular docking simulations have also been performed for rutin and reported binding energies ranging from −11.33 to −8.21 kcal/mol, with the simulation performed using different Mpro PDB entries (6LU7, 6Y84 and 6W63). Docking also indicated the formation of different hydrogen bonds in each simulation, which mainly include residues His41, Phe140, Cys145, His163 and Glu166. Moreover, the suitability of rutin for further development as a potential antiviral compound is reinforced by its favorable pharmacokinetic profile and stability in complex with Mpro, as resulted from molecular dynamics simulation [108,113,115-118]. Finally, quercetagetin, a flavonol structurally related to quercetin, has been found to have good inhibitory effect against the protease, with an IC50 of 1.24 μM [105]. The binding mode and structure of reported flavonoids, flavanones and their derivatives is presented in Fig. 10.

Gallic acid is a small, hydroxybenzoic acid which despite having a low docking score (-4.52 kcal/mol, as opposed to −9.22 kcal/mol for positive control N3), showed to hinder the cytopathic effect in infected Vero E6 cells with an EC50 of 108 μg/mL [116]. Various esters of gallic acid with flavan-3-ols have also provided promising results in in silico and in vitro studies. More specifically, epigallocatechin-3-O-gallate, gallotheacinn 3-O-gallate and epicatechin-3-O-gallate exhibited the same binding energy of −8.7 kcal/mol and comparable IC50 values in a fluorescent substrate assay, equal to 7.51, 6.38 and 5.21 μM respectively, while catechin-3-O-gallate showed a slightly lower binding affinity, with a binding energy of −8.3 kcal/mol, but lower IC50 of 2.98 μM [119]. In a similar in vitro assay performed by Jang et al. [120], epigallocatechin gallate showed an IC50 of 7.58 μg/mL, as well as no cytotoxicity in HEK293T cells up to a concentration of 40 μg/mL. Its auto-oxidation products were also reported to be active, whereas it showed an additive effect with theaflavin, with a coefficient of drug interaction (CDI) of 0.93. Both compounds are found in tea, green and black tea respectively, and theaflavin alone had an inhibitory effect against the protease with an IC50 of 8.44 μg/mL.

In the category of flavonoids and derivatives, naringenin has been reported to inhibit the enzyme by 50% at a concentration of 92 μM, while showing indications of good bioavailability and drug-likeness properties. Molecular docking simulations with the Glide software resulted in a binding energy of −7.83 kcal/mol, as well as hydrogen bond interaction with residues Tyr54 and Thr190 [121]. The same study calculated a slightly higher binding energy (-7.56 kcal/mol) but lower IC50 value (74 μM) for apigenin-7-O-glucoside, reporting also more interacting residues which form hydrogen bonds, namely Thr26, Met49, Glu166, Gln189, Thr190. Sennoside B and 2,3,4,5,6-pentahydroxybenzophenone are included in the aforementioned study, yielding lower binding energies (-9.01 and −8.34 kcal/mol respectively) but slightly higher IC50 values (104 and 102 μM, respectively), while establishing Glu166 as a common hydrogen bond forming residue.

Another flavanol included in various studies is curcumin, a major constituent of Curcuma longa. It has shown in vitro inhibitory effect by reducing the activity of the protease to 28.1% at a concentration of 75 μg/mL [122], while more information regarding its binding has been available through molecular docking simulations. Simulation with Autodock Vina resulted in a binding energy of −7.1 kcal/mol and hydrogen bonds with residues Gly143 and Ser144 [123], while the use of Glide gave a binding energy of −8.09 kcal/mol and hydrogen bonds with residues Gly143, Leu141, Glu166, Pro168, Gln189 and Thr190 as an output [124]. The α,β-unsaturated ketone group present in the molecule provides probable cause to investigate the possibility of covalent binding since it can act as a Michael acceptor. More specifically, covalent docking with CoVDock performed by Teli et al. [125] resulted in a binding energy of −7.03 kcal/mol, highlighting Gly143 as a hydrogen bond contact, as in the previously mentioned works, along with Thr26.

The major compounds identified in a Chinese herbal extract mixture known as Shuanghuanglian preparation, chlorogenic acid, baicalin and baicalein, also showed inhibitory effect demonstrated by IC50 values as low as 39.48, 6.41 and 0.94 μM, respectively [116]. When tested in Vero E6 cells, baicalin and baicalein exhibited EC50 values of 27.87 μM and 2.94 μM, respectively. Chlorogenic acid has been tested in other works as well [107,126], and its inhibitory effect is projected on its IC50 value, that equals 360 μg/mL, even though molecular docking simulations have resulted in a binding energy considerably lower (−7.18 kcal/mol) as opposed to the positive control (inhibitor N3, −9.22 kcal/mol) [116]. Baicalein has been co-crystallized in complex with M pro (PDB ID: 6M2N) and the co-crystallized structure revealed hydrogen bond interactions with residues Leu141, Gly143, Ser144, His163 and Glu166. Baicalin and baicalein are also major compounds of Scutellaria baicalensis and have been reported to have IC50 values of 83.4 and 0.39 μM in a different study [105]. The same study further evaluated the antiviral activity of baicalein in Vero cells and calculated an EC50 value of 2.92 μM. Other compounds found in the Shuanghuanglian preparation have shown inhibitory activity against M pro, including Scutellarein (IC50 = 3.02 ± 0.11 μM), Forsythoside A (IC50 = 3.18 ± 0.12 μM), Forsythoside B (IC50 = 2.88 ± 0.13 μM), Forsythoside E (IC50 = 6.68 ± 0.22 μM), Forsythoside H (IC50 = 10.17 ± 0.39 μM), Forsythoside I (IC50 = 5.47 ± 0.1 μM) and Isoforsythoside (5.85 ± 0.06 μM) [103]. Scutellarein, in particular, yielded an IC50 of 5.80 μM in a colorimetric substrate enzyme assay [105] while its glucoside has been reported to have better binding affinity to the protease than native inhibitor N3 (-9.3 kcal/mol as opposed to −8.93 kcal/mol) in in silico molecular docking simulations [127].

Triterpenes are another category of compounds that have provided indications of anti-M non- effects. More specifically, betulinic acid, betulin, ursolic acid and maslinic acid, all found in Olea europaea leaves extract among other plants, were evaluated in vitro through a fluorescent substrate cleavage assay, and showed encouraging results, portrayed by the calculated IC50 values of 14.55, 89.67, 12.57 and 3.22 μM. The fact that betulinic acid had considerably higher activity compared to betulin indicates the importance of the carboxyl group at C-17 for the interactions of the molecule with the protease [128]. Ursolic acid in particular has also been included in several virtual screening studies, often
Fig. 11. Binding mode and structure of phenylethanoid glycosides (Forsythoside A-Acteoside) and pentacyclic triterpenoids (Betulinic acid-Glycyrrhizin) with in vitro demonstrated inhibitory activity in the active site of SARS-CoV-2 Mpro. Catalytic residues are colored (His41: green, Cys145: yellow), residues participating in hydrogen bonds are depicted as yellow dashes. The receptor-ligand complex was produced by docking simulations using the software YARASA Structure, replicating the binding mode represented in the relevant publication (Available in Table 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
as a constituent of *Ocimum sanctum*, with binding energies ranging from $-8.88$ to $-8.52$ kcal/mol, as provided by Autodock Vina and Autodock 4.2. Binding of the molecule is attributed to the conventional active site of the protease in two studies, designating hydrogen bonds with residues Thr24, Leu141 and Ser144 [129,130].

Another study mentions formation of hydrogen bonds with residues Arg131, Lys137, Asp197, Thr198, Thr199, Tyr237, Asn238, Tyr239, Leu272, Gln273, Gly275, Met276, Leu286 and Leu287, suggesting binding of ursolic to another site [131].

**Glycyrrhizin** is a triterpenoid saponin with an EC$_{50}$ value of 0.44 mg/mL, as calculated from an antiviral assay performed in Vero E6 cells by van de Sand et al. [132]. A fluorescent substrate assay showed 70.3 % reduction of enzymatic activity at an inhibitor concentration of 30 $\mu$M and complete inhibition at 2000 $\mu$M. Glycyrrhizin has also been investigated in various docking studies, resulting in binding energies between $-9.57$ and $-7.9$ kcal/mol, while it has also shown *in silico* indication of inhibitory effect against other viral proteins including the spike protein, the heli-

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**Fig. 12.** Binding mode and structure other natural compounds with *in vitro* demonstrated inhibitory activity in the active site of SARS-CoV-2 Mpro. Catalytic residues are colored (His41: green, Cys145: yellow), residues participating in hydrogen bonds are depicted as yellow dashes. 2: 2,3,4,5,6-pentahydroxybenzophenone; 3: 24-methylcholesta-7-en-3β-ol. The receptor-ligand complex was produced by docking simulations using the software YARASA Structure, replicating the binding mode represented in the relevant publication (Available in Table –3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Table 4
Natural compounds highlighted in molecular docking studies for their potential inhibitory effect against SARS-CoV-2 Mpro.

| Compounds                                          | Source1 | Software      | PDB2 | Binding energy (kcal/mol) | H-bonds                  | Reference |
|----------------------------------------------------|---------|---------------|------|---------------------------|--------------------------|-----------|
| Flemichin A                                        | –       | Autodock      | 6LU7 | –8.9                      | Arg188                   | [149]     |
| Delta-Oleanolic acid                               | –       | Vina 4.2      |      | –8.9                      | Thr26, Glu166            |           |
| Emodin 1-O-beta-D-glucoside                        | –       |               |      | –8.7                      | Leu141, Asn142, Cys145, His163, Glu166, Pro168, Gin189 Thr190 |           |
| Procyandin A2                                      | Grape, strawberry, persimmon, cranberry, blueberry, cacao, green tea | Autodock | 6LU7 | –9.2                      | Gly143                   | [119]     |
| Epigallocatechin                                   | tea     | Vina          |      | –7.7                      | Glu166                   |           |
| Gallicatechin                                       |         |               |      | –7.6                      | Glu166                   |           |
| Epicatechin                                         |         |               |      | –7.5                      | Glu166, Gin189, Thr190   |           |
| Epiazelechin                                        |         |               |      | –7.5                      | Leu141, Glu166           |           |
| Afzelechin                                          |         |               |      | –7                        | Leu141, Glu166           |           |
| Mangiferin                                          | Mangifer indica | Autodock | 6LU7 | –8.4                      | His141, Leu141, Asn142, Gly143, Ser144, Cys145, Arg188, Thr190, Gin192 | [107]     |
| Kaempferol                                          | Mangifer indica, Moringa oleifera | Vina |      | –7.8                      | Leu141, Ser144, Gin189   |           |
| Lupeol                                              | Mangifer indica | Vina |      | –7.6                      | –                        |           |
| Nimboline                                           | Azadirachta indica | –       |      | –7.6                      | different site           |           |
| Ellagic acid                                        | Mangifer indica, Moringa oleifera | –       |      | –7.3                      | His141, Arg188, Thr190   |           |
| Gedunin                                             | Azadirachta indica | –       |      | –7.3                      | Asn142                   |           |
| Catechin                                            | Mangifer indica, Moringa oleifera | –       |      | –7.2                      | Glu166, Asp187, Thr190, Gin192 |           |
| Nimbandin                                           | Azadirachta indica | –       |      | –7.1                      | Thr26, Gly143            |           |
| Epicatechin                                         | Mangifer indica, Moringa oleifera | –       |      | –7                        | Ser144, His163,Gin189    |           |
| Nimbineene                                          | Azadirachta indica | –       |      | –6.5                      | Asn142, Gly143           |           |
| Hanpinin                                            | Zinger officinal | –       |      | –8.3                      | Asn142, Met165           |           |
| Methyl 3,4,5-trihydroxybenzoate                     | Rhus spp (sumac)  | Molegro | 6LU7 | –22.6                     | Phe140, Leu141, Ser144, Cys145, His164 | [126]     |
| (Z)-1-(2,4-Dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-hydroxyprop-2-en-1-one | Vina |      |      | –21.83                   | Leu141, Ser144, Cys145, His164, Asp187, Gin192 |           |
| 3,7-Dihydroxy-2-(4-hydroxyphenyl) chroman-4-one     |         |               |      | –17.21                    | Tyr54, Leu141, Ser144, His163, Glu166, Asp187, Gin192 |           |
| 2-(3,4-Dihydroxyphenyl)-3,5-dihydroxy-7-methoxy-4H-chroman-4-one | Vina |      |      | –15.57                    | Tyr54, Gly143, Ser144, Cys145, Glu166, Gin192 |           |
| (Z)-2-(3,4-Dihydroxybenzylidene)-6-hydroxybenzo[1,3]dibenzo[4,1]dioxin chroman-4-one | Vina |      |      | –14.31                    | Leu141, Ser144, Cys145, His164, Asp187, Gin192 |           |
| 3,5,7-Trihydroxy-2-(4-hydroxyphenyl) chroman-4-one  | Uncaria tomentosa (Cat’s claw) | Autodock | 6LU7 | –13.34                    | Tyr54, Leu141, Gly143, Ser144, Cys145, Glu166, Asp187, Gin192 | [151]     |
| Cadamine                                            | Phyllanthus amarus | Vina 1.1.2 |      | –8.6                      | Phe140, Ser144, Cys145, His163, Gin189, Thr190, Gin192 |           |
| Speciophyline                                       |         | Vina          |      | –8.1                      | Gly143                   |           |
| Geraniin                                            | Andrographis paniculata | Autodock | 6LU7 | –9.3                      | Leu141, Gly143, Ser144, Cys145, Thr190 | [114]     |
| Squalene                                            | Olea europaea | Autodock | 6W63 | –6.2                      | –                        | Not described |
| Theaflavine-3’-‘digallate                           | Black tea | Autodock 4.2.6. | 6LU7 | –12.41                    | Thr26, His142, Thr54, Cys145, His164 | [153]     |
| Hypericin                                           | Hypericum perforatum | –       |      | –11.17                    | Asn142, Cys145, His164, Glu166 |           |
| Robustaflavone                                      | Rhus succedane | –       |      | –10.92                    | Thr26, His163            |           |
| (+)-Solenolide A                                    | Haliotis laevigata | –       |      | –10.82                    | –                        |           |
| Hesperedin                                         | Citrus spp., Mentha spp., Linaria vulgaris | Autodock | 6LU7 | –8.3                      | Phe140, Ser144, Cys145, Glu166 | [146]     |
| Rhoifolin                                           | Rhus succedane, Citrus spp., Thalab purpureus, Lycopersicon esculentum, Cynara scolymus, Musa spp., Vitis vinifera | Autodock | 6LU7 | –8.2                      | –                        |           |
| Pectolinarin                                        | Cirsiun spp., Linaria vulgaris | –       |      | –8.2                      | Ser144, Cys145, His163, Gin189, Glu166 |           |
| Nabiximols                                          | Cannabis spp. | –       |      | –8                        | Asn142, Met165, Thr190   |           |
| Quercetin-3-vicianoside                             | –       | Autodock      | 6LU7 | –8.3                      | Thr26, Leu141, Gly143, Ser144, His163, Gin189 | [154]     |
| Absinthin                                           | Artemisia Absinthium | –       |      | –8.2                      | His 163                  |           |
| Delphinidin 3-O-glucoside                           | Phaseolus Vulgaris | –       |      | –8                        | Thr26, His142, Gly140, Pro168, Gin189, Thr190 |           |
| Petunidin 3-O-glucoside                             | Phaseolus Vulgaris | –       |      | –8                        | Thr26, His142, Phe140, Met165, Thr190 |           |
| Quercetin 3-glucuronicid-7-glucoside                | Phaseolus Vulgaris | –       |      | –7.9                      | His141, Phe140, Gly143, Glu166 |           |
| Chrysoeriol 8-C-glucoside                           | Phaseolus Vulgaris | –       |      | –7.9                      | Thr26, Gly166, Thr190    |           |
| Piperolactam A                                      | Piper Longum | –       |      | –7.7                      | Leu141, Gly143, Ser144, Cys145, His163 |           |
| Compounds                               | Source                     | Software       | PDB$^2$ | Binding energy (kcal/mol) | H-bonds Reference                  | Reference                  |
|-----------------------------------------|----------------------------|----------------|---------|---------------------------|-----------------------------------|----------------------------|
| Oleanolic acid                          | Ocimum Gratissimum         | AutoDock       | 6M71    | 5.87                       | His163, Thr190                    | [145]                      |
| Schafoside                              | Phaseolus Vulgaris         | Vina           | 6Y84    | 6.76                       | Gly143                            | [146]                      |
| Riboflavin                              | Curcuma Longa              | AutoDock       | 6LU7    | 5.78                       | Leu141, Ser144                    | [147]                      |
| Echinacoside                            | Echinacea-angustifolia     | Glide (XP protocol) | 5R82    | 4.59                       | Thr25, Cys44                      | [148]                      |
| Quercetagentin-7-glucoside             |                            |                |         |                           |                                   |                            |
| Levan N                                 |                            |                |         |                           |                                   |                            |
| Inulin from chicory                     |                            |                |         |                           |                                   |                            |
| Hydroxycinnamic acid                   | Aviscenia officinalis      | Vina           | 6Y2G    | 4.41                       | His163, Thr190                    | [149]                      |
| Phenethyl alcohol                      |                            |                |         |                           |                                   |                            |
| Dihydroarteminisine                    |                            |                |         |                           |                                   |                            |
| caffeic acid phenethyl ester           |                            |                |         |                           |                                   |                            |
| Withanone                               |                            |                |         |                           |                                   |                            |
| Thalimonine                             | Thalictrum simplex         | AutoDock 4.2   | 6Y2F    | 5.59                       | Gly143                            | [150]                      |
| Sophaline D                            | Sophora alopecuroides     | Glide          | 6LU7    | 5.76                       | Gly143                            | [151]                      |
| Isorhamnetin                           | Horchata herbal infusion  | GOLD           | 6Y2C    | 4.87                       | His163, Thr190                    | [152]                      |
| Benzoyl acid, 2-(ethylthio)-, ethyl ester |                            |                |         |                           |                                   |                            |
| Mearnsin                                | Manilkara hexandra         | Not specified  | 6LU7    | 5.79                       | Not described                     | [153]                      |
| Quercetin 3-O-β-D-glucoside            |                            |                |         |                           |                                   |                            |
| Bondicellpin D                          | Caesalpinia minax         | AutoDock4.2    | 6Y2F    | 5.91                       | Gly143                            | [154]                      |
| Caesalmin B                             |                            |                |         |                           |                                   |                            |
| 11-Oxa-dispiro[4.0.4.1]undecan-1-ol     |                            |                |         |                           |                                   |                            |
| Azetidin-2-one 3,3-dimethyl-4-(1-aminooxy) |                    |                |         |                           |                                   |                            |
| Lorazepam, TMS derivative              |                            |                |         |                           |                                   |                            |
| Jacein                                  | Crepis sancta              | AutoDock       | 6LU7    | 5.55                       | Gly143                            | [155]                      |
| (6S,9R)-Roseoside                       |                            | Vina           |         |                           |                                   |                            |
| Pandoletin                              |                            |                |         |                           |                                   |                            |
| Pachypodol                              |                            |                |         |                           |                                   |                            |
| Chrysopleninet                          |                            |                |         |                           |                                   |                            |
| Isorhamnetin-3-O-β-D                   | Calendula officinalis     | AutoDock       | 6LU7    | 4.86                       | Not described                     | [156]                      |
| Calendoflaside                          |                            | Vina           |         |                           |                                   |                            |
| Narcssin                                |                            |                |         |                           |                                   |                            |
| Calendulaglycoside B                   |                            |                |         |                           |                                   |                            |
| Calenduloside                           |                            |                |         |                           |                                   |                            |
| Legalon                                 | Silybum marianum           | AutoDock 4.2   | 6M71    | 4.87                       | Not described                     | [157]                      |
| Robinetinidol                           | Acacia mearnsii            | Vina           |         |                           |                                   |                            |
| Mesquit                                 | Prosopis juliflora        |                |         |                           |                                   |                            |
| Hesperidin                              |                            | SwissDock server | 6Y84    | 9.02                       | Thr24, Thr25, His41, Thr45, Ser46, Cys145 | [158]                      |
| Berberine                               | Tinospora cordifolia       | AutoDock       | 6LU7    | 7.18                       | Thr25, Ser46, His163              | [159]                      |
| b-Stigosteryl ferulate                  |                            | AutoDock       | 6LU7    | 8.73                       | Not described                     | [160]                      |
| Cordifoliside                           | Tinospora cordifolia       | AutoDock       | 6LU7    | 7.56                       | His163, Thr190                    | [161]                      |
| Fagaronine                              | Fagaraz anthoxyloides      | AutoDock 4.2   | 6LU7    | 5.79                       | Glu14, Gly71, Lys97, Ser121       | [162]                      |
| Isoboldine                              | Corydalisca, Glaucium flavum, Peumus bolo | AutoDock 4.2 | 6LU7    | 5.49                       | Glu14, Gly71, Lys97, Ser121       | [163]                      |
| Sageone                                 | Salvia officinalis         | AutoDock 4.2   | 6LU7    | 5.98                       | Cys145, His163                    | [164]                      |
| Lycorine                                | Clivia minata             | Vina           |         |                           |                                   |                            |
| Wogonin                                 | Scutellaria baicalensis | AutoDock       | 6LU7    | 5.62                       | Thr199, Tyr239, Leu271            | [165]                      |
| Epicatechingallate                      | Green tea                 | AutoDock       | 6LU7    | 7.09                       | Phe140, Gly143, Ser144, Cys145, His163 | [166]                      |
| Gallatechin-3-gallate                   |                            |                |         |                           |                                   |                            |
| (continued on next page)
| Compounds                  | Source \(^1\) | Software | PDB\(^2\) | Binding energy \((kcal/mol)\) | H-bonds | Reference |
|---------------------------|--------------|----------|-----------|-------------------------------|---------|-----------|
| Epicatechin               |             |          |           |                               |         |           |
| Catechin gallate          |             | AutoDock 4.2 | 7BQY     | –7.1                          |         | Leu141, Ser144, His163, Gln192 |
| Deacetylnomilin           |             | –        |           |                               |         | Ser144, His163, Gln192         |
| Ichangin                  |             | –        |           |                               |         | His163    |
| Nomilin                   |             | –        |           |                               |         | Asn142, Ser144, Gln166         |
| β-Amyrin                  |             | –        |           |                               |         | Gln192    |
| Hyperoside                | Neem         | AutoDock | 6LU7      | –8.6                          | Thr26, Gln192 | Leu141, Ser144, His163, Arg188, Thr190, Gln192 |
| 9-Hederin                 | Nigella sativa | –       |           |                               |         | His163, Gln166, Gln189         |
| Nimbaflavone              | Neem         | –        |           |                               |         | His163    |
| Epigallocatechin          | Camellia sinensis | –  |           |                               |         | Leu141, Ser144, Cys145, His163, Glu166 |
| Catechin                  |             | –        |           |                               |         | Thr26, Gln189                      |
| Piperine                  | Piper nigrum | –        |           |                               |         | Thr25, Ser144, Cys145          |
| Echinocystic acid diacetate | Luffa cylindrica | – |           |                               |         | Glu166    |
| Hypericin                 |             | –        |           |                               |         | Leu141, Asn142, Gln166         |
| Pseudohypericin           |             | Vina     |           |                               |         | Gln192    |
| Cyanidin-3-Glucoside      |             | –        |           |                               |         | Thr26, Leu141, Gly143, Gln166, Asp187, Gln189 |
| Glabridin                 |             | –        |           |                               |         | Thr26, Gln166                      |
| Amentoflavone             | Torreya nucifera | – |           |                               |         | Thr26, Gln166                      |
| Ginkgetin                 |             | –        |           |                               |         | Thr26, Asn142                      |
| 3’-(3-methylbut-2-enyl)-3’,4’,7’-trihydroxyflavane | Broussonetia papyrifera | – |           |                               |         | Thr26, Gln166                      |
| Broussonachalcone A       |             | –        |           |                               |         | Thr26, Gln166                      |
| Kazinol F                 |             | –        |           |                               |         | Thr26, Gln166                      |
| Kazinol J                 |             | –        |           |                               |         | Thr26, Gln166                      |
| Papyriflavonol A          |             | –        |           |                               |         | Thr26, Gln166                      |
| Broussoflavan A           |             | –        |           |                               |         | Thr26, Gln166                      |
| Heptafuhalol A            | Sargassum spinuligerum | – |           |                               |         | Thr26, Gln166                      |
| Phlorethopentafuhalol B   |             | –        |           |                               |         | Thr26, Gln166                      |
| Pseudopentafuhalol C      |             | –        |           |                               |         | Thr26, Gln166                      |
| Phlorethopentafuhalol A   |             | –        |           |                               |         | Thr26, Gln166                      |
| Hydroxypentafuhalol A     |             | –        |           |                               |         | Thr26, Gln166                      |
| Pentaphlorethol B         |             | –        |           |                               |         | Thr26, Gln166                      |
| 8,8’-Bieckol              |             | –        |           |                               |         | Thr26, Gln166                      |
| Apigenin-7-O-neohesperidoside |             | –        |           |                               |         | Thr26, Gln166                      |
| Luteolin-7-rutinoside     |             | –        |           |                               |         | Thr26, Gln166                      |
| 6,6’-Bieckol              |             | –        |           |                               |         | Thr26, Gln166                      |
| Dieckol                   |             | –        |           |                               |         | Thr26, Gln166                      |
| Pseudotheonamide D        |             | –        |           |                               |         | Thr26, Gln166                      |
| Aeruginosin 98B           |             | –        |           |                               |         | Thr26, Gln166                      |
| Resinoside B              |             | –        |           |                               |         | Thr26, Gln166                      |
| Pentaphlorethol A         |             | –        |           |                               |         | Thr26, Gln166                      |
| Tunichrome An2            |             | –        |           |                               |         | Thr26, Gln166                      |
| Pseudotheonamide C        |             | –        |           |                               |         | Thr26, Gln166                      |
| Berbamine                 | Berberis asiatica | – |           |                               |         | Thr26, Gln166                      |
| Oxyacanthine              |             | –        |           |                               |         | Thr26, Gln166                      |
| 1-(3-(2,5,9-trimethyl-7-oxo-3-phenyl-7H-furo[3,2-g]chromen-6-y1)propanoyl)piperidine-4-carboxamide (ZINC02123811) | – | – |                               |         | Thr26, Gln166                      |
| Palmatine                 |             | –        |           |                               |         | Thr26, Gln166                      |
| Sauchinone                | Saururus chinensis | – |           |                               |         | Thr26, Gln166                      |
| Diosmetin                 | Citrus limon | –        |           |                               |         | Thr26, Gln166                      |
| Apigenin                  |             | –        |           |                               |         | Thr26, Gln166                      |
| Luteolin                  |             | –        |           |                               |         | Thr26, Gln166                      |
| Eriodictoyl               |             | –        |           |                               |         | Thr26, Gln166                      |
| Spinacetin                |             | –        |           |                               |         | Thr26, Gln166                      |

\(^1\) Source: \(^2\) PDB code
| Compounds                          | Source1                        | Software          | PDB2            | Binding energy (kcal/mol) | H-bonds                      | Reference         |
|-----------------------------------|--------------------------------|-------------------|-----------------|--------------------------|------------------------------|-------------------|
| Taraxerol                         | Clorodendrum spp              | Autodock          | 6LU7            | –8.4                     | –                            | [174]             |
| Friedelin                         | Vina                           |                   |                 | –7.9                     | –                            |                   |
| Stigmasterol                      |                                 |                   |                 | –7.7                     | –                            |                   |
| Demethoxyguaiavine                | Strynch nos-ux-vomica          | Autodock          | 6Y2G            | –10.1                    | Arg188, Thr190               | [175]             |
| Sterchondlavine                   | Vina                           |                   |                 | –9.9                     | Arg188, Thr190               |                   |
| NB-Methyllyconicadatine           |                                 |                   |                 | –9.6                     | Thr26, Ser142, Glu166        |                   |
| Bis-nor-dihydrotroferine          |                                 |                   |                 | –9.4                     | Asn142                       |                   |
| Strychnosine                      |                                 |                   |                 | –9.1                     | Arg188                      |                   |
| Guianensine                       |                                 |                   |                 | –8.8                     | Ser46, Arg188                |                   |
| Vomicine                          |                                 |                   |                 | –8.7                     | Gly143, Ser144              |                   |
| 10-Hydroxy-icajine                |                                 |                   |                 | –8.6                     | His41, Leu141, Gly143, Cys145|                   |
| N-methyl-sec-pseudo-beta-coulbrine|                                 |                   |                 | –8.3                     | His41, Phe140, Gly143, His163,Glu166 |                   |
| Steryomicine                      |                                 |                   |                 | –8.3                     | Leu141, Gly143, Ser144      |                   |
| Postularin 3                      | Family Aplisinae               | MOE               | 6MO3            | –7.58                    | Ser46, Met49, Asp187, Gln192, Ala194, Thr169, Gln189 | [109]             |
| Garnatin                          |                                 | Glide             | 6LU7            | –7.74                    | His41, Asn142, Gly143, Gln189| [124]             |
| Robinetin                         |                                 |                   |                 | –7.51                    | Thr26, His41, Met165, Asp187 |                   |
| Vitexin                           | Morina olifera                 | Autodock          | 6W63            | –8.4                     | His163, Glu166, Asp187       | [176]             |
| Kaempferol-3-O-rutinoside         |                                 |                   |                 | –8.2                     | His41, Glu166, Leu167, Arg188, Thr190 |                   |
| Neandrographolide                 | Andrographis paniculata        | Autodock          | 6LU7            | –7.1                     | Phe140, Leu141, Ser144, His163, Glu166 | [177]             |
| Psi-taraxasterol                  |                                 | Autodock          | 6LU7            | –8.5                     | Met49, Cys145, Met165        | [148]             |
| Kazinol T                         | Broussonia kazinoki            | Piper             | 6Y7M            | –14.36                   | His41, Gly143, Thr190        | [178]             |
| Butyrocolactone I 3-sulfate       | Aspergilus terreus             | algorithm         |                 | –13.85                   | His41, Gly143, Cys145, Glu166|                   |
| Ebenurifan III                    | Onyrobotis ebonoides           |                   |                 | –13.56                   | Asn46, Met165                |                   |
| Paulowniones A                    | Paulownia tomentosa            |                   |                 | –13.47                   | Ser144, Glu189              |                   |
| 3,5,7-Trihydroxy-8-(3-Methoxy-3-Methylbutyl)-2-(4-Methoxyphenyl) Chromen-4-One | | | | | |
| Schizoleanone B                   |                                 |                   |                 | –12.72                   | Not described               |                   |
| Praeruptorin B                    |                                 |                   |                 | –12.7                    | Not described               |                   |
| NPG67197                          |                                 |                   |                 | –12.59                   | Not described               |                   |
| Variecolorin G                    |                                 |                   |                 | –12.58                   | Not described               |                   |
| 2-Hydroxyargin A                  |                                 |                   |                 | –12.23                   | Not described               |                   |
| Todicoumarquinone                 |                                 |                   |                 | –12.05                   | Not described               |                   |
| (4-Hydroxy-3-Methoxy-2,5-Dimethylphenyl)-3-Formyl-2,4-Dihydroxy-6-Methylbenzoate | | | | | |
| Withanolide R                     | Withania somnifera             | Autodock 4.2.     | 6LU7            | –9.63                    | Not described               | [179]             |
| 27-Deoxy-14-hydroxywithafarin A   |                                 |                   |                 | –10.8                    | Not described               |                   |
| Nimolicinol                       |                                 |                   |                 | –10.09                   | Not described               |                   |
| 17-Hydroxywithafarin              |                                 |                   |                 | –10.08                   | Not described               |                   |
| Urso-deoxycholic acid             | Ipomea obscura                 | Glide 5.5         | 6LU7            | –7.11                    | Ser46, Phe140               | [180]             |
| Demeclocycle                      |                                 |                   |                 | –6.81                    | Gly143, Glu166              |                   |
| Tetracycline                      |                                 |                   |                 | –5.95                    | Glu166                      |                   |
| Chlorotetraycline                 |                                 |                   |                 | –4.72                    | Thr26, Leu141, Gly143, Ser144|                   |
| Ethyl iso-allocholate             |                                 |                   |                 | –4.42                    | Thr26, Gln189               |                   |
| Agathisflavone                    | Anacardium occidentale         | Autodock          | 5R81            | –8.2                     | Ar40, Pro52, Asp187         | [181]             |
| Rubusic acid                      | Pedalium murex                 | Vina              |                 | –8.1                     | Not described               |                   |
| Solanocapine                      | Solanum nigrum                 |                   |                 | –7.9                     | Not described               |                   |
| Chlorogenicin                     | Solanum torvum                 |                   |                 | –7.7                     | Not described               |                   |
| Lupeol                            | Carica papaya and Azadirachta indica | | | –7.7 | Not described | |
| Cyanin                            | Zingiber officinalis           |                   |                 | –7.7                     | Thr26, Ser46, Gln166        |                   |
| 3-O-trans-cafeeylormaic acid      | Terminalia chebula             |                   |                 | –7.7                     | Not described               |                   |
| Luteolin 7-O-(6-malonylglucoseide)| Vitex negundo                  |                   |                 | –7.7                     | Not described               |                   |
| Aeguside                          | Vitex negundo                  |                   |                 | –7.6                     | Not described               |                   |
| Luteolin 7-O-beta-D-glucoside     | Vitex negundo                  |                   |                 | –7.6                     | Not described               |                   |
| Azefin                            | Euphorbia Hirta                | Autodock          | 6LU7            | –9.3                     | Tyr54, Thr190, Leu141, Ser14, Glu166 | [110]             |
| Phlorogcinol                      | Hypericum perforatum L.        | Vina              |                 | –9.3                     | Arg188, Gln189              |                   |
| Myricetin-3-O-rutinoside          | Limoniatiastrum Gynynianum      |                   |                 | –9                      | Leu141, Gly143              |                   |
| Trich 7-moheperidioside           | Chamaenerhum humile L          |                   |                 | –8.5                     | Glu166, Thr26, Ser144, Cys145|                   |
| Silybin                           | Silybum marianium              |                   |                 | –8.3                     | Glu166, Ser144, Cys145      |                   |
| Silychristin                      | Silybum marianum L             |                   |                 | –8.3                     | Arg188, Asn142              |                   |
| Germicanoide                      | Costus speciosus               | Autodock 4.2.     | 6LU7            | –7.4                     | His163                      | [182]             |
| Andrograpain                      | Andrographis paniculata        |                   |                 | –7.37                    | Not described               |                   |
| Hetsisone                         | Aconitum heterophyllum         |                   |                 | –7.37                    | Not described               |                   |
| Costunolide                       | Costus speciosus               |                   |                 | –7.3                     | Not described               |                   |
| 14-deoxy-14,15-didehydroandrographolide | | | | | |

(continued on next page)
| Compounds                                    | Source1                  | Software | PDB2       | Binding energy (kcal/mol) | H-bonds Reference |
|----------------------------------------------|--------------------------|----------|------------|---------------------------|-------------------|
| Palmatine                                    | Tinosoria cordifolia     | Glide    | 6LU7       | −7.12                     | Not described     |
| Hetsine                                      | Aconitum heterophyllum   |          |            | −7.1                      | Not described     |
| 14-deoxy-11,12-didehydroandrographolide      | Andrographis paniculata |          | 706        | −7.06                     | Not described     |
| Isoaorbol                                    | Gmelina arborea          |          |            | −6.97                     | Not described     |
| Serratin                                     | Clerodendrum serratum    |          |            | −6.95                     | Not described     |
| Piperamide                                   | Piper nigrum             |          |            | −6.84                     | Not described     |
| Bampine                                      | Piper nigrum             |          |            | −6.77                     | Not described     |
| Abscisic acid                                | Pterocarpus marsupium    |          |            | −6.68                     | Not described     |
| Gnetinol                                     | Gmelina arborea          |          |            | −6.47                     | Not described     |
| Laurotetanin                                 | Lutea glutinosa          |          |            | −6.38                     | Not described     |
| Phyllanthidine                               | Phyllanthus emblica      |          |            | −6.36                     | Not described     |
| Cepharadione                                 | Piper longum             |          |            | −6.06                     | Not described     |
| Pogopryrone                                  | Pogostemon cablin        |          |            | −6.03                     | Not described     |
| Boldine                                      | Lutea glutinosa          |          |            | −5.86                     | Not described     |
| Vomifolol                                    | Sida acuta               |          |            | −5.59                     | Not described     |
| N-isobutyl-(2E,4Z,8Z,10E)-dodecataeranemide   | Anacyclus pyrethrum      |          |            | −5.5                      | Not described     |
| Delphinidin 3,5-diglucoside                  | Pomegranate              | Glide    | 6LU7       | −12.2                     | Leu141, Asn142, Cys145, His164, Glu166, Thr190 | [127] |
| 3,5-Di-O-galloylshikimic acid                | –                        | Glide    | 6LU7       | −10.3                     | Asn142, Gly143, His163, Glu166, Gln189, Thr190 |
| Aviculin                                     | Polygonum aviculare,Rhododendron aureum, Taxillus kaempferi | Glide | 6LU7 | −9.6 | Cys145, His164, Glu166, Thr190 |
| Scutellarein 7-glucoside                    | Verbena officinalis L, Buddleja madagascariensis Lam, Plantago asiatica L. Polygonum odoratum | Glide | 6LU7 | −9.3 | Cys145, His163, Glu166, Glu192 |
| 3,8’-biapigenin                              | Hypericum perforatum     | Vina     | 6W63       | −10.4                     | Thr26, Ser46, Asn142, His163, Glu166 |
| Methyl amentollavone                         | Selaginella sinensis, Ginkgo biloba, Cupressaceae spp. | Glide | 6LU7 | −10 | His41, Met49, Glu166, Leu167 |
| Podocarpus flavone A                         | Podocarpus macrophyllus, Garcinia spp. | Glide | 6LU7 | −10 | His41, Met49, Glu166, Leu167 |
| Kaempferol-3-robiniobioside                  | Piper nigrum, Annona coriacea | Glide | 6LU7 | −9.8 | Thr26, Tyr54, Leu141, Asn142, Cys145, His163, Glu166 |
| Isoginkgetin                                 | Ginkgo biloba            |          |            | −9.8                      | His41, Met49, Glu166, Leu167 |
| Theassinensin B                             | Camelia sinensis         |          |            | −9.8                      | Not described     |
| 3,5 digalloylepicatechin                     | Camelia sinensis         |          |            | −9.8                      | Not described     |
| Neothoeflavin 3-gallate                     | Camelia sinensis         |          |            | −9.7                      | Not described     |
| Quercetin 3-O-xylosyl glucuronide            | apruche fruit, blackberry, and raspberry | Glide | 6LU7 | −9.5 | Not described     |
| Vitamin D2                                   | –                        | Glide    | 6LU7       | −9.5                      | Not described     |
| Albamn F                                     | Morus alba               |          |            | −9.4                      | Leu141, Cys145, Glu166, Asp187 |
| Bianthaquinone                               | Polygonaceae, Rhamnaceae, Rubiaceae, Fabaceae, Xanthorrhoeaceae | Glide | 6LU7 | −9.4 | Not described     |
| Isoqueretin                                  | Mangifera indica, Rheum rhabarbarum, Annona reticulata, camelia sinensis | Glide | 6LU7 | −9.3 | Not described     |
| Withastramonomide                            | Withania somnifera       | Glide    | 6LU7       | −8.9                      | Not described     |
| Luteolin 7-O-b-glucopyranoside               | Withania somnifera       | Glide    | 6LU7       | −9.54                     | Asn142, Gly143, Cys145, Glu166 |
| Acacetin 7-O-b-rutinose                      | Withania somnifera       | Glide    | 6LU7       | −8.54                     | Gly143, Cys145 |
| Isoacetoside                                 | Withania somnifera       | Glide    | 6LU7       | −8.46                     | Thr26, His41, Met49, Gly143, His164, Met163, Glu166, Gln189 |
| Luteolin (+)- 3a-O-b-glucopyranoside         | –                        | Glide    | 6LU7       | −8.34                     | Asn44, Met49, Leu141, Cys145 |
| Amphaniculoside A                           | Cynamortis sinensis, Glu166 | Glide | 6LU7 | −7.59 | Cys44, Met49, Leu141, Cys145 |
| (-)-Lamnirolis in 3a-O-b-glucopyranoside     | –                        | Glide    | 6LU7       | −7.56                     | Asn142, Gly143, Glu166 |
| 2”-3”-Diacetyl martynoside                  | –                        | Glide    | 6LU7       | −7.45                     | Thr26, Met49, Asn142, Cys145, Met163 |
| Isomortynoside                              | –                        | Glide    | 6LU7       | −7.02                     | Asn49, Met142, Met165 |
| Cinnamantannin B2                           | Cinnamomum zeylenicum    | Autodock | 6LU7    | −6.68                     | Asn142, Met165, Glu166, Leu167 |
| Cinnamomum zeylenicum                       | Cinnamomum zeylenicum    | Autodock | 6LU7    | −10                     | Glu166, Gln189 |
| Cyanin                                      | Allium sativum           | Vina     | 6LU7       | −9.4                      | Asn142, Gly166, Thr190 |
| Withanoside V                               | Withania somnifera       | Autodock | 6LU7    | −10.32                    | Asn48, Arg40, His141, Gln189, Thr190 |
| Somniferine                                 | Withania somnifera       | Autodock | 6LU7    | −9.62                     | Leu141, His164, Thr24, Glu166, Asn142, Phe 140, His163 |
| Tinosoricide                                | Tinospora cordifolia     |          |            | −8.1                      | Leu141, Gly143 |
| Vicenin                                     | Ocimum sanctum           |          |            | −8.97                     | Glu166, Pro168, Gln189, Thr190 |
| Isororientin 4’-O-glucoside 2”-O-p- hydroxybenzoate | Ocimum sanctum          |          |            | −8.55                     | Arg40, Tyr54, Arg105, Arg188 |
| Capsazepine                                 | Capsicum annuum L.       | Autodock | 6LU7    | −8.8/-7.0                | His41, Cys145, Glu189 |
| Aronadendin                                 | Allium cepa L.           | Autodock | 6LU7    | −8.7/-7.9                | Glu166 |
| Leucopelargonidin                           | Allium cepa L.           | Autodock | 6LU7    | −7.8                     | Glu166, Gln189 |
Table 4 (continued)

| Compounds                                                                 | Source                  | Software       | PDB2 | Binding energy (kcal/mol) | H-bonds Reference |
|---------------------------------------------------------------------------|-------------------------|----------------|------|---------------------------|-------------------|
| Astragaline                                                               | Opuntia ficus-indica   | Vina           | 6Y84 | –6.7                      | Phe140, Glu166    |
| Isorhamnetin                                                              | Vina                    | Autodock       | –7.9 | –                        |                   |
| Isorhamnetin 3-O-glucoside                                                | Vina                    | –7.3           | Thr26, Asn142, Gln189      |
| 3-O-caffeyl quinic acid                                                   | Paederus littoralis     | Autodock       | –7.5 | Thr24, Thr26, His41, Leu141, Asn142, Gly143, Gln189 |
| Quercetin 5,4’-dimethyl ether E, E                                         | Halymenia durvillei     | GOLD           | 6LU7 | 27.4/5.0                  | Not described     |
| Z-1,3,12-nonadecatriene-5,14-diol                                         | Marine-derived actinomycetes | Autodock       | 21.7 | –3.8                      |                   |
| Cholest-5-En-3-Ol (3.Beta.)-                                              | Withania somnifera      | Glide          | LE7  | –8.96                     | Thr24, Thr25, Thr26, His163Glu166 |
| Solanolin                                                                 | Solaum genus            | Glide XP       | LE7  | –10.3                     | Leu141, His164, Glu166 |
| Procyanadin A3                                                           | –                       | protocol       | –12.86| –                        |                   |
| Procyanadin A4                                                           | –                       | –10.01         | Glu166, Pro168, Gln189, Thr190 |
| Procyanadin B4                                                           | itch pericarp, grape seeds | –9.94         | Leu141, Asn142, Gly143, Gln166 |
| Hypercin                                                                  | Hypericum perforatum    | –9.56          | Leu141, His164, Glu166, Gln189 |
| Procyandin                                                               | –                       | –9.21          | Leu141, Asn142, Glu166, Thr190 |
| Astragalain                                                               | Allium ursinum, Allium sativum, Cassia alata, Cuscuta chinensis, Phytolacca americana | –9.12         | Leu141, Thr190             |
| Salicin                                                                   | –                       | –8.45          | Not described             |
| Emodin-8-glucoside                                                        | –                       | –8.21          | Not described             |
| Hinokiflavone                                                             | –                       | –8.13          | –                        |
| Procyanadin C2                                                           | –                       | –8.11          | Not described             |
| Indican                                                                   | –                       | –8.08          | Not described             |
| Chebulic acid                                                             | –                       | –8.08          | Not described             |
| Amentoflavone                                                             | –                       | –7.98          | –                        |
| (−)-Catechin gallate                                                      | –                       | –7.96          | Not described             |
| Fisetin                                                                   | –                       | –7.94          | –                        |
| 18,β-Glycyrrhetinic acid                                                  | –                       | –9.19          | Not described             |
| Rhodioli                                                                 | Luffa cylindrica        | PyRx 0.9.4     | 6LU7 | –7.54                     |                   |
| Lucyside H                                                                | Luffa cylindrica        | PyRx 0.9.4     | 6LU7 | –7.47                     | Not described     |
| 3-O-β-p-Glucopyranosyl-oleanolic acid                                     | –                       | –7.29          | –                        |
| 3-O-β-p-Glucopyranosyl-spinasterol                                        | –                       | –7.13          | –                        |
| Anisotine                                                                 | Justicia adhatoda       | Autodock       | 6LU7 | –8.4                      | Gly143            |
| Amarogentin                                                               | Sertia chirata          | Vina           | –8.0 | H141, Glu166              |
| Adhatodine                                                                | Justice adhatoda        | –7.9           | Thr26                     |
| Beta-carotene                                                             | Ocimum sanctum          | –7.8           | –                        |
| Mangiferin                                                                | Sertia chirata          | –7.8           | Leu141, Gly143, Ser144, His164, Glu166, Thr190 |
| Eugenol                                                                  | Ocimum sanctum          | –7.6           | Glu166                    |
| Vasicoline                                                                | Justice adhatoda        | –7.4           | –                        |
| Vasicolinone                                                              | Justice adhatoda        | –7.3           | Gly143, Ser144, Cys145    |
| Caryophyllene                                                             | Ocimum sanctum          | –7.1           | Pro168, Gln189            |
| Crocin                                                                    | Crocus Sativus L.       | Autodock       | 6LU7 | –8.2                      | Phe3, Arg4, Lys5, Arg131, Arg133, Thr135, Lys137, Thr199 |
| Digitoxigenine                                                            | Nertum Oleander         | Vina           | –7.2 | Gln110, Asp135            |
| β-Eudesmol                                                                | Lauris Nobilis L        | –7.1           | Thr111                    |
| Bergenin                                                                  | Dictyophora indusiata   | Autodock 4.2   | 6LU7 | –7.86                     | Gly143, Ser144, His163, Glu166, Gln189 |
| Quercitrin                                                                | Geasstrum triplex       | –10.2          | Tyr54, His163, Thr190, Gln192 |
| Dihydroaeraminsinin                                                       | Cytatus stercerus       | MOE            | –7.2 | Gly143, Ser144, Cys145    |
| Dihydro-onnamide A                                                        | Marine sponges (Theonella and | 6Y2G | –10.2 | –                        |                   |
| Onnamide C                                                                | Trachycladius genera    | 2019.012       | 9.60 | Pro168                    |
| Pseudo-onnamide A                                                         | –                       | –9.81          | Thr26, Gly170             |
| Theopederin G                                                             | Theonella marine sponges| –8.45          | Gly143, His164            |
| Pederin                                                                   | Paederus littoralis     | –7.95          | Asn142, Gly143, His164    |
| Pyranongrin A                                                             | Paederus littoralis     | Autodock       | –7.5 | Leu141, Asn142, Gly143, Ser144, Cys145, His163, Glu166, Gln189 |
| Citriquinichroman                                                         | Penicillum citrinum     | Autodock       | –14.7| Thr26, Asn142, Gly143, Cys145, Glu166, Asp187, Arg188, Gln193, Thr190, Gln192 |
| Holylrine B                                                               | Marine-derived actinomycetes | –14.5         | Leu141, Asn142, Gly143, Ser144, Cys145, His163, His164, Glu166, Pro168, Asp187, Arg188, Gln189, |

(continued on next page)
case and RdRp. It is interesting that Muhsen et al. [132] report Asn238 and Asp289 as residues participating in hydrogen bonds with the compound, suggesting its binding affinity to a different site of the protease than the active site [123,133,134].

**Vanicosides A and B** are two phenylpropanoid glycosides detected in plants *Reynoutria japonica* and *Reynoutria sachalinensis* that have been found to inhibit Mpro in *vitro* with IC50 values of 23.10 and 43.59 μM, respectively. In *silico* analysis of their binding to the protease using the software GOLD reveals a higher docking score than inhibitor N3 (115.78 and 129.7 as opposed to 86.56) and hydrogen bonds with residues Thr26, Cys145, Asn142, Gly143, His164 and Thr190. The binding mode of acteoside in the active site of Mpro is more likely to be non-covalent, despite the presence of a η,β-unsaturated ester moiety, which could theoretically act as a covalent warhead [121]. Another work describes the results of both covalent and non-covalent docking simulations for acteoside and reports docking scores of −11.98 and −6.91 kcal/mol, respectively [125]. A third study including the compound conducted non-covalent docking and calculated a higher binding energy (−8.33 kcal/mol) with quite different interactions, showing formation of hydrogen bonds with major residues Gly143, Cys145, His164 and Glu166 [136].

Another category of compounds with confirmed anti-SARS-CoV-2 Mpro activity by *in vitro* assays is procyanidins. More specifically, procyanidin B2 appeared to block S1, S1' and S2 subsites of the protease and form hydrogen bonds with residues Gly143, Cys145 and Glu166 in a molecular docking simulation performed using Autodock Vina. The binding energy was calculated to be as low as −9.2 kcal/mol. Its inhibitory effect was confirmed *in vitro* with an IC50 of 75.31 μM calculated through a fluorescent substrate assay [119]. A derivative of the compound, Procyanidin B2 3,3'-di-O-gallate, has been tested in a different study, with a similar type of assay, and resulted in approximately 37% inhibition at a concentration of 100 μM. Procyanidin C1 reduced enzymatic activity to 77.7% at the same concentration [135]. Other procyanidins, namely procyanidin A3, A4, B4 and C2, have displayed very promising binding energies in an *in silico* study (~12.86, −10.01, −9.94 and −8.11 kcal/mol). It is worth mentioning that all four compounds have a more favorable binding energy than inhibitor N3, which was used as positive control and had a binding affinity of −7.93 kcal/mol. It is worth to notice that three of four procyanidins showed higher binding affinity than the *in vitro* documented active procyanidin B2, for which a binding energy of −8.56 kcal/mol was calculated in the same study [125].

**24-methylcholesta-7-en-3β-ol** is a phytosterol, detected among many plant sources including *Zingiber officinale*, while also being the most abundant sterol in *Polyporus sulfureus*. When its inhibitory effect was evaluated with a FRET-based assay, the compound caused 75% enzyme inhibition at 200 μg/ml, while the positive control GC376 resulted in 77% inhibition at 100 μM. Moreover, molecular dynamics simulation indicated good stability of its complex with Mpro, as well as hydrogen bonding with residue Cys44 [137].

**Punicalagin** is a large, complex natural compound found in abundance in pomegranate. It reduced the activity of Mpro by half at a concentration of 6.19 μg/ml in a fluorescent substrate assay, while it displayed synergy with zinc sulfate, reducing the activity of the protease 24% more than punicalagin alone, when the two compounds were at concentrations of 10 μg/ml and 3 mg/ml, respectively [138].

The binding mode and structure of reported triterpenoids and phenylethanoid glycosides is presented in Fig. 11, while other compounds are presented in Fig. 12. In addition, there have been...
7. Plant extracts with inhibitory activity against SARS-CoV-2 M<sub>pro</sub>

Apart from pure natural compounds, extracts containing various constituents have been evaluated for their overall inhibitory effect against M<sub>pro</sub>. The inhibition in such cases is often attributed to the synergistic effect of the major bioactive compounds in the extract. A study performed by Guijarro-Real et al. [122] tested various plant extracts for their ability to inhibit M<sub>pro</sub> in a FRET-based assay and underlined mustard seeds, wall rocket and turmeric extracts as plant extracts with high inhibitory potential. More specifically, the IC<sub>50</sub> values calculated were 15.74 μg/mL for the turmeric extract, 128.1 μg/mL for the mustard seeds extract and 257.4 μg/mL for the wall rocket extract. Commercial curcumin, present in turmeric extracts, showed inhibitory activity against M<sub>pro</sub>, as mentioned previously. However, the inhibitory effect of the compound combined with the fact that reported concentrations of curcumin in turmeric powder do not exceed 3%, suggest that the activity of the extract is not due to curcumin alone, but also due to other components of the extract. Moreover, allyl isothiocyanate, a hydrolysis derivative of sinigrin, which naturally occurs in wall rocket and mustard extracts, demonstrated strong inhibition of M<sub>pro</sub> with an IC<sub>50</sub> of 41.43 μg/mL, providing an encouraging lead for further investigation. Celery leaves, parsley, oregano, aloe vera leaf and wasabi powder extracts also exhibited moderate inhibitory activity, resulting in reduction of the activity of the enzyme to 35.8–54.8% at a concentration of 500 μg/mL.

The traditional Chinese patent medicine, Shuanghuanglian preparation, which is being used for treatment of acute respiratory tract infections has been also investigated in in vitro assays. A FRET assay was used to determine the inhibitory effect of the medicine in the form of oral liquid, as produced from three different companies, and resulted in the calculation of IC<sub>50</sub> values of 0.90 ± 0.044, 0.064 ± 0.011 and 0.076 ± 0.007 μL/mL respectively. When tested in Vero E6 cells, the three oral liquids resulted in an EC<sub>50</sub> of 1.20 ± 0.18, 1.07 ± 0.04 and 0.93 ± 0.19 μL/mL respectively. The high content of the oral liquids in baicalin (12.72 to 17.52 mg/mL) as opposed to baicalin (0.06–0.22 mg/mL) leads to the conclusion that baicalin is mainly responsible for the inhibitory effect of the preparation against SARS-CoV-2 M<sub>pro</sub> [103].

Plants Reynoutria japonica and Reynoutria sachalinensis have been used in Chinese traditional medicine to combat upper respiratory tract infections, too. Both their acetone and butanol extracts have been evaluated for their SARS-CoV-2 M<sub>pro</sub> inhibitory activity, yielding encouraging results. Overall, R. sachalinensis showed better inhibitory effect, with IC<sub>50</sub> values of 9.42 and 4.03 μg/mL for the acetone and butanol extracts respectively, compared to 16.90 and 7.88 μg/mL for R. japonica. Evidently, the butanol extracts performed better compared to the acetone ones. The higher inhibitory activity was attributed to the presence of more procyanidins and phenylpropanoid disaccharide esters [135].

Low IC<sub>50</sub> values were also provided by the extract of Cuphea ignea. A crystal violet assay was used to evaluate both the ethanolic extract of the plant and a self-nanoemulsifying formulation containing oleic acid, Tween 20 and propylene glycol with improved solubility. The respective IC<sub>50</sub> values were almost identical, 2.47 and 2.46 μg/mL [139]. Comparable IC<sub>50</sub> values were also calculated for the flavonoid-rich fraction of the aqueous extract of Salvadora persico (IC<sub>50</sub> = 8.59 μg/mL), the aqueous extracts of green (IC<sub>50</sub> = 8.9 μg/mL) and black tea (IC<sub>50</sub> = 10.0 μg/mL) and Terminalia chebula (IC<sub>50</sub> = 8.8 μg/mL), as well as the ethanol extract of Scutellaria baicalensis (IC<sub>50</sub> = 8.52 μg/mL) calculated through a fluorescent, colorimetric and casein substrate inhibition assays [105,140,141]. Lastly, the aqueous extract of licorice is reported to have an antiviral effect at a concentration of 2 mg/mL in Vero E6 cells infected with a viral load of 100 times the 50% tissue culture infective dose/mL [132].

The information on the inhibitory effect of the plants extracts is presented in Table 5.

Table 5: Plant extracts with tested inhibitory activity against SARS-CoV-2 M<sub>pro</sub> by in vitro assays.

| Plant                     | Type of extract  | Major constituent(s)                                   | IC<sub>50</sub> (μg/mL) | Method          | Reference |
|---------------------------|------------------|--------------------------------------------------------|--------------------------|-----------------|-----------|
| Curcuma longa             | methanolic extract | Curcumin                                              | 15.74                    | FRET assay  [122] |           |
| Brasica nigra             | methanolic extract | Sinigrin, allyl isothiocyanate                         | 128.1                    |                 |           |
| Diplotaxis erucoides      | methanolic extract | Sinigrin, allyl isothiocyanate                         | 257.4                    |                 |           |
| Loniceria japonica, Scutellaria baicalesis, Forsythia suspense | commercial shuanghuanglian oral liquids | Chlorogenic acid, phillyrin, baicalin, baicailein,   | 0.064–0.090     | FRET assay  [103] |           |
| Reynoutria japonica       | butanol extract   | Proanthocynadinis, flavan-3-ols, phenylpropanoid disaccharide esters | 7.88                     | Fluorescent substrate assay  [135] |           |
| Reynoutria sachalinensis  | butanol extract   | Proanthocynadinis, flavan-3-ols, phenylpropanoid disaccharide esters | 4.03                     |                 |           |
| Cuphea ignea              | ethanolic leaf extract | p-Coumaric acid, Myricetin-3-O-rhamnoside, Gallic acid, Rutin, Siringic acid | 2.47                     | Crystal violet assay  [139] |           |
| Salvador A persico L      | FRF (flavanoid rich fraction) from the aqueous extract of the plant leaves and stems | Kaempferol and isorhamnetin glycosides | 8.59                     | Fluorescent substrate assay  [140] |           |
| Scutellaria baicalesis    | 70% ethanol extract | Baicalin, baicailein, wogonin, wogonoside             | 8.52                     | Colorimetric substrate enzyme inhibition assay  [105] |           |
| Camellia sinensis (green tea) | aqueous leaf extract | Thearubigine, queretin-3-O-rutinoside, hesperidin     | 8.9                      | Casein substrate enzymatic assay  [141] |           |
| Camellia sinensis (black tea) | aqueous extract | Thearubigine, queretin-3-O-rutinoside, hesperidin     | 10.0                     |                 |           |
| Terminalia chebula        | aqueous extract   | Not described                                          | 8.8                      |                 |           |
| Licorice                  | aqueous root extract | Glycyrrhizin                                          | 8.8                      |                 |           |
8. Conclusions

Overall, research has resulted in very promising leads regarding both the design of targeted drugs and the utilization of isolated natural compounds or crude plant extracts. The former are able to very efficiently inhibit \( M_{\text{pro}} \), even at micromolar concentration levels, while the latter, despite displaying an inhibitory effect at overall higher concentrations compared to the designed drugs, can open up various possibilities for valorization of biomass and developing alternative solutions for boosting immunity. In both cases, it is very encouraging that there are numerous effective candidates with high potential against \( M_{\text{pro}} \), while some also show indication of action against other viral proteins. Taking into consideration the high conservation observed in the sequences encoding \( M_{\text{pro}} \) among coronaviruses, many of these compounds have originated from research targeting the main proteases of SARS-CoV-1, MERS or other viruses. In a similar manner, the large number of data emerging from current research is not only useful for combating the ongoing pandemic, but also for laying foundations for ways to fight future viral outbreaks. In this context, it is important to point out that in silico methods play a major role in identifying potent hits, facilitating the study of structure–activity relationships and the prediction of suitable structural groups, the rapid screening of large number of candidates, as well as the investigation of the impact of potential mutations on the efficacy of these candidates. However, it is necessary that both the antiviral and the pharmacokinetic properties of these compounds are further investigated in vitro and in vivo, so as to determine whether they can be used as pharmaceutical products or functional foods, respectively.

CRediT authorship contribution statement

Io Antonopoulou: Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. Eleftheria Sapountzaki: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Ulrika Rova: Conceptualization, Methodology, Resources, Validation, Writing – review & editing. Paul Christakopoulos: Conceptualization, Methodology, Resources, Validation, Writing – review & editing.

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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