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Potential SARS-CoV-2 main protease inhibitors

Riddhidev Banerjee[1], Lalith Perera[2] and L.M. Viranga Tillekeratne[1]

[1] Department of Medicinal and Biological Chemistry, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, OH 43606, USA
[2] Laboratory of Genome Integrity and Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC 27709, USA

The coronavirus disease 2019 (COVID-19) pandemic has prompted an urgent need for new treatment strategies. No target-specific drugs are currently available for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), but new drug candidates targeting the viral replication cycle are being explored. A prime target of drug-discovery efforts is the SARS-CoV-2 main protease (M^{pro}). The main proteases of different coronaviruses, including SARS-CoV-2, SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), share a structurally conserved substrate-binding region that can be exploited to design new protease inhibitors. With the recent reporting of the X-ray crystal structure of the SARS-CoV-2 M^{pro}, studies to discover M^{pro} inhibitors using both virtual and *in vitro* screening are progressing rapidly. This review focusses on the recent developments in the search for small-molecule inhibitors targeting the SARS-CoV-2 M^{pro}.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic has left a mark in more than 180 countries, with more than 61 million cases worldwide and over 1.4 million deaths as of 27 November 2020 (Johns Hopkins Coronavirus Resource Center, https://coronavirus.jhu.edu/map.html). This has had a devastating effect not only on people's lives, but also on the entire global economy. Coronaviruses (CoVs) are large viruses containing a single-stranded RNA genome within an enveloping membrane that is covered in glycoprotein spikes. Animals such as bats host the largest variety of CoVs [1]. Although four genera of coronavirus exist (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*), our present concerns lie with the *Betacoronavirus* genus. This genus includes the severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and the COVID-19 causative agent SARS-CoV-2 [2]. SARS-CoV-2 has the distinction of aff ;ecting multiple organs and the central nervous system, and can cause respiratory problems with fatal consequences [3,4]. The information available indicates that SARS-CoV-2 is highly contagious, although not as fatal as SARS-CoV [5]. The *Betacoronavirus* genome encodes: (i) structural proteins such as the glycosylated spike (S) protein, which mediates host cell receptor recognition and host cell entry, and induces host immune responses; and (ii) non-structural proteins such as RNA-dependent RNA polymerase (RdRp), the CoV main protease [M^{pro}; also known as 3-chymotrypsin-like protease ($3CL^{pro}$)] and papain-like protease (PL^{pro}) [6]. During viral replication, the M^{pro} and PL^{pro} process the viral polyproteins, synthesized using the host cell translational machinery, to generate a functionally active viral replication complex for packaging within host cells [7] (Fig. 1). Hence, these proteases present attractive targets for small molecule inhibitors.

No target-specific drugs are currently available for SARS-CoV-2, and therefore strategies such as repurposing existing drugs are being investigated as a matter of urgency. Some common drugs that have been prescribed as therapeutic interventions for COVID-19 include a combination of lopinavir and ritonavir, ribavirin, chloroquine phosphate, hydroxychloroquine, arbidol, remdesivir, favipiravir and dexamethasone [8–10]. This review aims to showcase a general collection of recent theoretical and experimental work performed in search of agents specifically targeted at the SARS-CoV-2 M^{pro}.

Corresponding authors: Perera, L. (lalith.perera@nih.gov), Tillekeratne, L.M. Viranga (ltillek@utnet.utoledo.edu)

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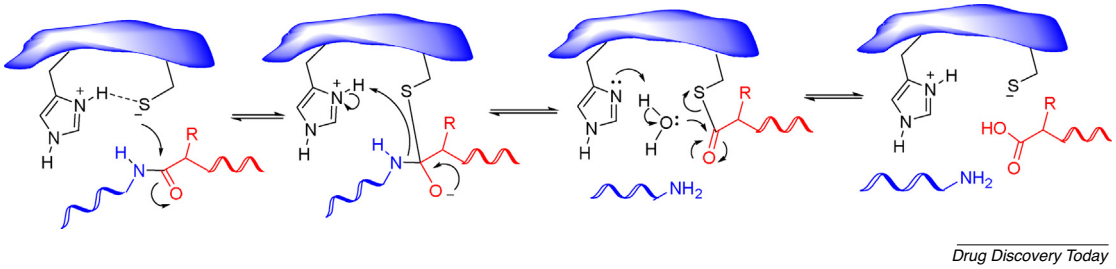

FIGURE 1
Mechanism of action of cysteine proteases.

Structure of the SARS-CoV-2 main protease

SARS-CoV-2 replication is mediated by a complex formed from two polyproteins that are translated from the viral RNA. These polyproteins are cleaved in at least 11 sites around the C-terminal and the central region by the action of the catalytic residues in M^{pro}, releasing the vital proteins required for viral replication [11]. The M^{pro} of SARS-CoV-2 comprises three domains (Fig. 2a); domain (residues 8–101), domain (residues 102–184) and domain (residues 201–303). The first two domains have an antiparallel β-barrel structure, whereas the third, with five α-helices, forms an antiparallel conglomerate connected to domain by a long loop region (residues 185–200). The M^{pro} of the SARS-CoV viruses has a Cys-His catalytic dyad (shown in solid fill in Fig. 2a), with the substrate-binding site located between domains and . A previous study established that CoV M^{pro}s share a structurally highly conserved substrate-recognition pocket, a promising target for drug design and development [12]. The recent discovery of new CoVs and the structural data of CoV M^{pro}s from different strains have provided ways to further examine this. The superposition of 12 crystal structures of M^{pro}s (SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-HKU1, BtCoV-HKU4, MHV-A59, PEDV, FIPV, 312 TGEV, HCoV-NL63, HCoV-229E and IBV) [12–20] revealed that all CoV M^{pro}s share the same substrate-binding region between domains and as a result of structure conservation [21].

All residues potentially interacting with substrates in the active site of SARS-CoV M^{pro} (namely Thr24, Thr25, His41, Cys44, Met49, Tyr54, Phe140, Asn142, Gly143, Cys145, His163, His164, Met165, Glu166, Leu167, Pro168, Asp187, Arg188, Gln189 and Thr190) are conserved in SARS-CoV-2 (Fig. 2b,c), and therefore SARS-CoV-2 is not expected to show a difference in catalytic activity from SARS-CoV [22]. However, six variations in the amino acid residue sequence are found in the catalytic site of MERS-CoV (Thr24Ser, Thr25Met, Met49Leu, Asn142Cys, His164Gln, Pro168Ala and Arg188Lys). SARS-CoV-2 and SARS-CoV M^{pro}s share 96% sequence identity (Fig. 2c). In fact, the structural features of the SARS-CoV-2 monomer are similar to other previously reported M^{pro}s [12–20].

The structural similarity of the active site of the SARS-CoV and SARS-CoV-2 M^{pro}s is remarkable (Fig. 2d). Except for the Ala46Ser mutation found near the active site, the other differing residues listed in Fig. 2c for the two SARS-CoV M^{pro}s are situated relatively far from the active site. The M^{pro} exists as a homodimer in solution, and this dimer form is said to be highly active compared to the monomer form [23]. This dimer interaction surface was well characterized previously for SARS-CoV [24]. The recent X-ray crystal structure of the SARS-CoV-2 M^{pro} dimer [25] (resolved with a bound α-ketoamide inhibitor) is shown in Fig. 2e, and this dimer interaction surface is similar to the one observed in the SARS-CoV counterpart. The dimer interaction surface is also located on the opposite side of the active site (Fig. 2e). The active site therefore can be specifically targeted for anti-CoV activity. Hence, based on sequence and structure alignments and functional identity of key residues, it should be possible to design broad spectrum inhibitors to target different M^{pro}s belonging to the same class [22,26,27].

SARS-CoV-2 main protease inhibitors

Prior to the evaluation of the SARS-CoV-2 M^{pro} crystal structure, and the resulting search for its possible inhibitors, the crystal structure of the SARS-CoV M^{pro} showed Michael addition of the Sγ-atom of the catalytic Cys145 to the pi-bond of the unsaturated ester group in the mechanistic inhibitor N1 (Fig. 3a), and a water molecule stabilized the inhibitor through hydrogen bonds to the carboxylate of the ester and residues Gly143 and Asn142 in the enzyme active site [12]. Zhu et al. used highly electrophilic peptidomimetic aldehydes as warheads to target Cys145 of the SARS CoV M^{pro} for inhibition [28], whereas Zhang *et al.* targeted SARS-CoV M^{pro} using α-ketoamide and Michael acceptor-based hybrid inhibitors [29]. An elaborate description of structure-based drug discovery for prior CoV M^{pro}s can be found in a review by Hilgenfeld [30]. To achieve covalent binding of inhibitors to the catalytic cysteine of the SARS-CoV M^{pro}, other types of peptidic and peptidomimetic inhibitors were synthesized with different electrophilic functional groups, such as halomethylketones, epoxyketones, nitriles and phthalhydrazide ketones. All of these compounds successfully inhibited SARS-CoV replication in cell culture. Taken together, the conserved active site domain between the SARS-CoV and SARS-CoV-2 M^{pro}s should enable inhibitors of the former to target the latter.

The first crystal structure of the SARS-CoV-2 M^{pro} was resolved in early 2020 in complex with a designed mechanistic inhibitor (ligand N3; Fig. 3a) to 2.1 Å resolution [21]. This structure of M^{pro} with the ligand N3 revealed several key features of the inhibitor–protein interactions. The ligand N3 in its extended form in the M^{pro} binding pocket gave rise to a wealth of information on the involvement of various residues. In the case of this inhibitor, a combination of hydrogen bonding (residues Phe140-A, Gly143-A, His163-A, His164-A, Glu166-A, Gln189-A and Thr190-A) and hydrophobic interactions (residues His41-A, Met49-A, Tyr54-A, Met165-A and Leu167-A) serve to nestle the molecule deeply in

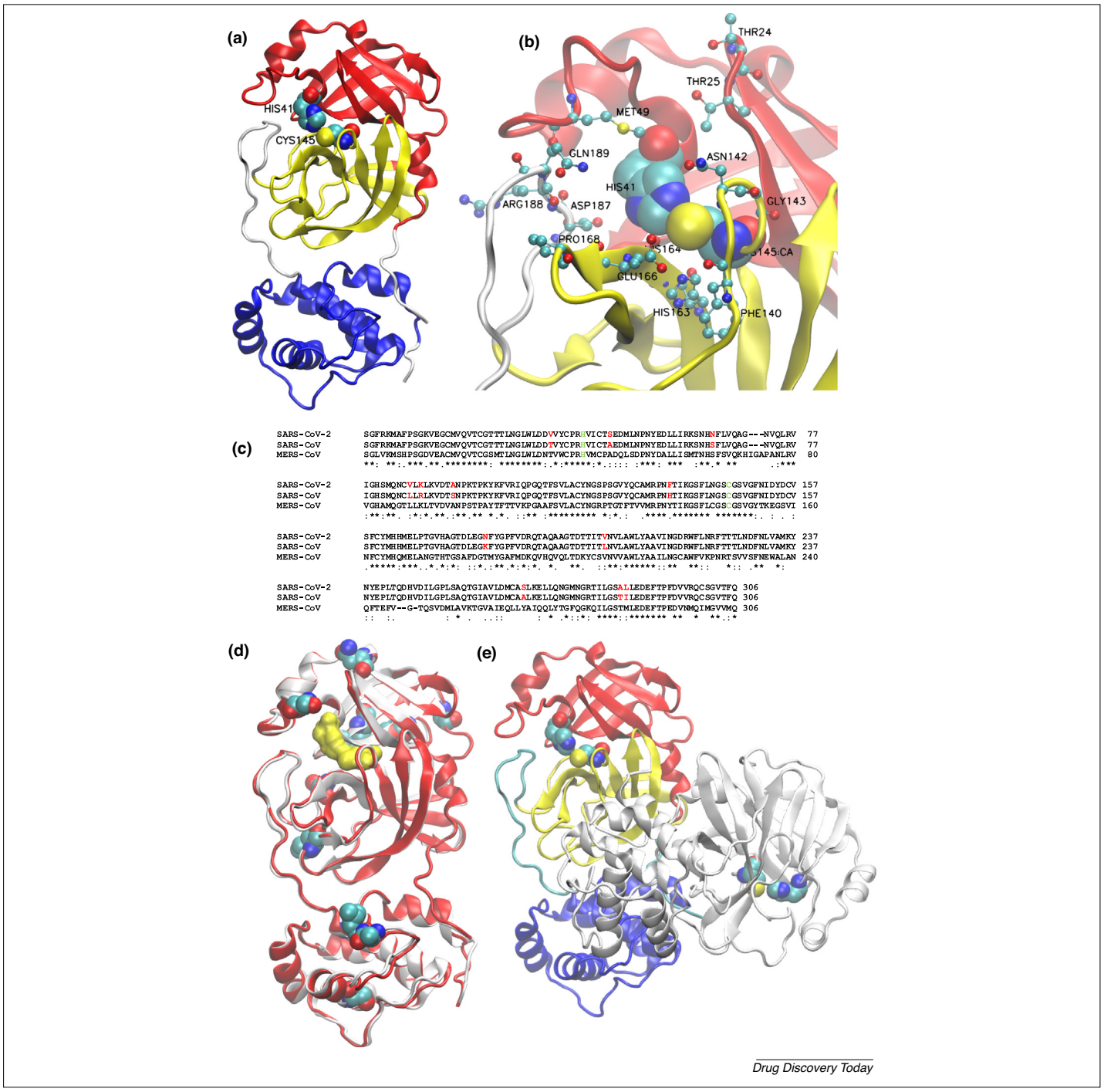

FIGURE 2

(a) The ribbon representation of the crystal structure of the SARS-CoV-2 M^{pro} from PDB ID: 6Y2F. Domains I, II and III are displayed in red, yellow and blue, respectively. The connection region between II and III is in white and the catalytic dyad residues (His41 and Cys145) are in solid spheres. **(b)** Protease active site residues that are involved in the inhibitor interactions. **(c)** Multiple sequence alignment of SARS-CoV-2 (Gene Bank ID: 045512.2), SARS-CoV (Gene Bank ID: NC004718.3) and MERS-CoV (Gene Bank ID: KT006149.2) using CLUSTAL W (1.83) [87]. Twelve residues that differ between SARS-CoV-2 and SARS-CoV are marked in red. The catalytic residues are marked in green. Active site residues are marked out with arrows. **(d)** Structure alignment between SARS-CoV in white (PDB ID: 2H2Z) and SARS-CoV-2 in red (PDB ID: 6LU7). Residues that differ between the two sequences are shown as solid spheres. The catalytic dyad residues, Cys145 and His41, are shown in yellow. **(e)** The dimer structure from PDB ID: 6Y2G. Domains I, II, and III of monomer A are in red, yellow and blue, respectively, whereas monomer B is in white. The catalytic dyad of each dimer is also shown. The dimer-interacting surface is situated on the opposite side of the active site.

the M^{pro} active site, locking it in position. Thereafter, nearly 100 crystal structures with bound inhibitors at the M^{pro} active site of SARS-CoV-2 were deposited in the PDB, showing the involvement of various active site residues in substrate binding. Those SARS-CoV-2 M^{pro} residues that can potentially interact with target molecules are shown in Fig. 3d. As noted here, preservation of the active site residues and the 3D structure of the site itself serve as key points for designing and developing target-specific inhibitors of

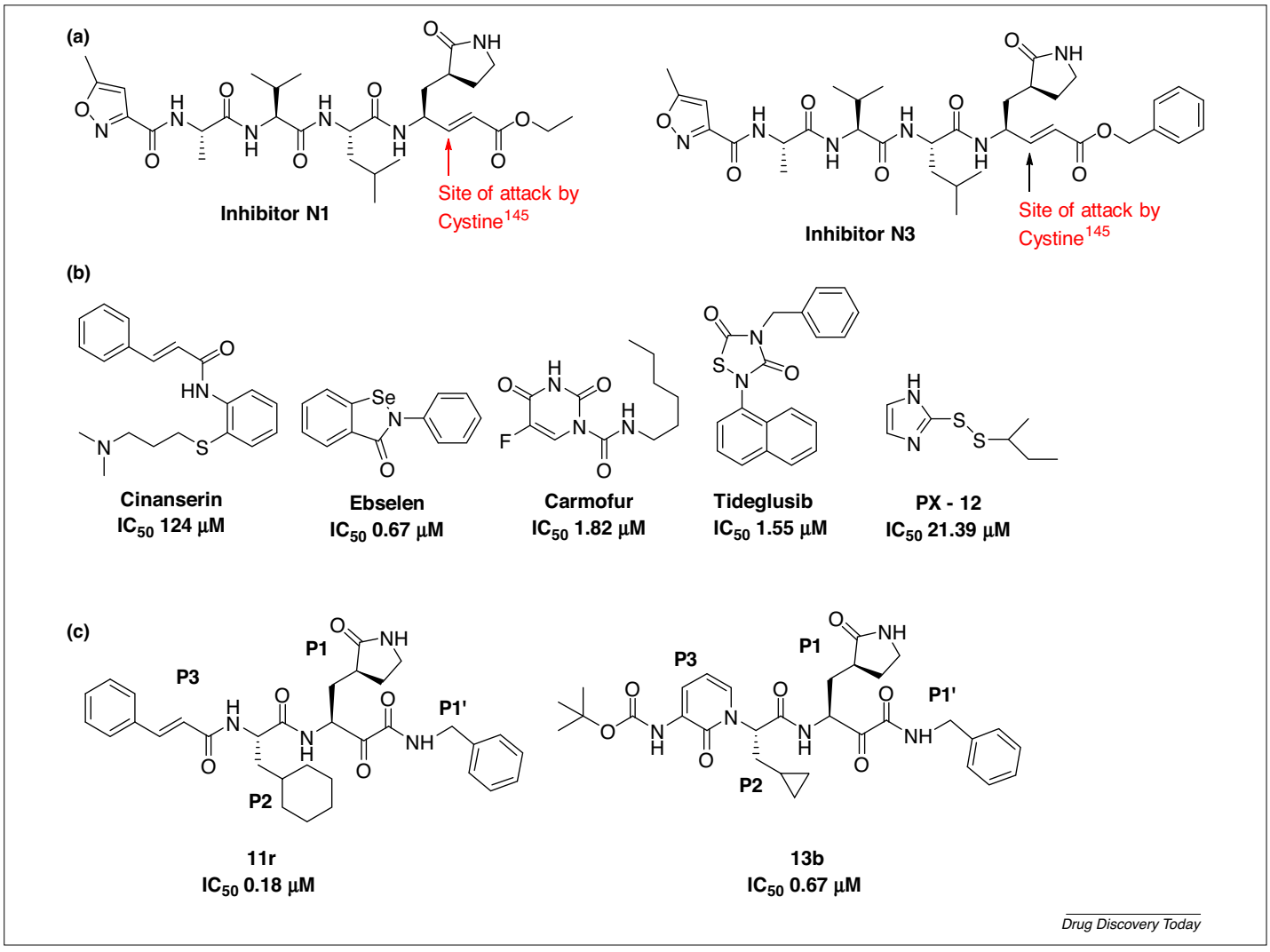

FIGURE 3

(a) Covalently bound inhibitors used for the SARS-CoV-2 M^{pro} (N3) and the SARS-CoV M^{pro} (N1) [21]. **(b)** Potential inhibitors binding covalently to Cys145 of the SARS-CoV-2 M^{pro} [21]. **(c)** α-Ketoamides display SARS-CoV-2 M^{pro} inhibitory activity. Compared with Michael acceptors, the thiohemiacetal intermediate formed is stabilized by an additional hydrogen bond with the catalytic center of the protease. Highlighted here are compounds 11 r and 13b, with their peptide regions designated P1, P1′, P2 and P3 [25].

the SARS-CoV-2 M^{pro}, based on the information already garnered from the studies carried out in selecting proper inhibitors for the M^{pro} of SARS-CoV. In the following section, we summarize the main inhibitors selected from numerous recent studies of the SARS-CoV-2 M^{pro}.

Jin *et al.* [21] observed that the Michael addition of the catalytic Cys145 of the protease to inhibitor N3 results in irreversible inhibition of the SARS-CoV-2 M^{pro}, in a similar way to inhibitor N1 (Fig. 3a) with the SARS-CoV M^{pro}. The enzyme inhibition took place in a time-dependent manner in a two-step irreversible inactivation mechanism. The inhibitor first associates with the enzyme non-covalently, before forming a stable covalent bond. Docking studies showed that several hydrophobic, van der Walls and hydrogen bonding interactions stabilize the inhibitor molecule within the substrate-binding pocket. Because the inhibitor was potent and the inhibition of the enzyme was rapid, it was not possible to measure the dissociation constant K_i and the inactivation rate constant k_3 for covalent bond formation. A pseudo second-order inactivation constant of $11{,}300 \pm 800\ M^{-1}s^{-1}$ was determined, suggesting a low dissociation constant and rapid covalent inactivation by the Michael acceptor, a feature that is useful in avoiding cross-reactivity with other enzymes and drug side effects. It had a CC_{50} value > 133 μM. Hence, based on similarity in interactions between N1 and the SARS-CoV M^{pro}, covalent bonding to Cys145 made it an important criterion for the selection of inhibitors.

Screening of more than 10,000 compounds, consisting of approved drugs, drug candidates in clinical trials and other pharmacologically active compounds, by structure-based virtual screening and a fluorescence energy transfer (FRET)-based assay revealed seven compounds that inhibited M^{pro} with IC_{50} values in the range of 0.67–21.4 μM. Two of them, disulfiram and carmofur (Fig. 3b), are US Food and Drug Administration (FDA)-approved drugs, whereas ebselen, tideglusib, shikonin, PX-12 and TDZD-8 are in clinical trials or in preclinical development. Of these, ebselen was the strongest inhibitor of M^{pro}, with an IC_{50} value

of 0.67 μM. Using tandem mass spectrometry analysis, ebselen, PX-12 and carmofur were found to covalently bind to the catalytic Cys145 of the enzyme. The relatively higher inhibitory activity of ebselen might be due to a relatively higher rate of covalent interaction, although the authors suggest that it could be a result of ebselen inhibiting the enzyme by non-covalent binding. Of the non-covalent binders, molecular docking studies showed that tideglusib, shikonin and disulfiram could fit well inside the substrate-binding pocket of the enzyme. In a cell-based antiviral activity assay, ebselen demonstrated strong antiviral activity with an EC_{50} of 4.67 μM, compared with an IC_{50} value of 16.77 μM for N3. Ebselen is an organoselenium compound with low cytotoxicity [31–34] and is suggested to have clinical potential for COVID-19 treatment. The study also found cinanserin (Fig. 3b) as a potential antiviral drug lead. Virtual screening showed that it snuggles into the substrate-binding pocket of the SARS-CoV-2 M^{pro} and inhibits the enzyme with an IC_{50} of 125 μM. It had moderate antiviral activity, with an EC_{50} of 20.61 μM. It might be possible that the observed higher antiviral activity of cinanserin compared with its enzyme inhibitory activity is due to the vinyl amide group functioning as a weak Michael acceptor and undergoing slow covalent interaction with the enzyme. However, the authors did not observe evidence to suggest such interaction. Similarly, a question arises if disulfiram which possesses a thiol reactive disulfide moiety like PX-12 would have formed a covalent adduct.

Zhang et al. designed several α-ketoamides as potential SARS-CoV-2 M^{pro} inhibitors [25]. As expected, compound 11 r (shown in Fig. 3c with peptide regions P1, P1′, P2 and P3) exhibited a low IC_{50} value of 0.18 μM. When the α,β-unsaturated amide functionality was hidden within an amino pyridone in the P3 skeleton to increase metabolic stability, followed by *tert*-butoxycarbonyl (Boc) protection, the resulting IC_{50} value was 2.39 μM. Replacement of the cyclohexyl group with a smaller cyclopropyl group (compound 13b in Fig. 3c) immediately lowered the IC_{50} value back to 0.67 μM, but subsequent Boc-deprotection made the compound completely inactive, and therefore it was concluded that the Boc group is necessary for biological activity. The crystal structure of compound 13b with the SARS-CoV-2 M^{pro} contained a thiohemiacetal link between the α-keto group and Cys145. The thiohemiacetal oxygen located in the P1′ region of 13b is stabilized by hydrogen bonding to His41, whereas the adjacent benzyl amide oxygen accepts a hydrogen bond from the main-chain amides of Gly143, Cys145 and partly Ser144, forming an oxyanion hole that is characteristic of this protease. This type of substrate has the advantage of its electrophilic warhead being stabilized by two hydrogen bonds, as opposed to one-hydrogen-bond interaction with a standard Michael acceptor. This work represents an important follow-up to the previous study of α-ketoamides as SARS-CoV M^{pro} inhibitors [29].

Dai et al. used structure-based drug design to develop antiviral drug candidates targeting the SARS-CoV-2 M^{pro}. They synthesized two peptidomimetic aldehydes, compounds 11a and 11b in Figure 4a, that displayed excellent inhibitory activity of the SARS-CoV-2 M^{pro}, with IC_{50} values of 53 nM and 40 nM, respectively [35]. X-ray crystal structure characterization showed that Cys145 forms a thiohemiacetal linkage with the aldehyde, with the hemiacetal oxygen being stabilized by hydrogen bonding with the backbone of residues Cys145 and Gly143. Stabilization by hydrogen bonding and hydrophobic interactions throughout the molecule results in high inhibitory activity. Although the cyclohexyl group of compound 11a produced extensive hydrophobic interactions with the surrounding side chains of Met49, Tyr54, Met165 and Asp187, it was noted that the replacement of the $-CH_2$-cyclohexyl group with an aryl moiety resulted in the $-CH_2C_6H_4F$ group of compound 11b deviating from its original position on alignment of 11a and 11b, owing to rotation about the $-CH-CH_2-$ bond. This rotation brought the aryl group close to His41, Met49, Met165 and Val186, creating additional hydrophobic contacts, thus lowering the IC_{50} value. This study complements the previous work of Zhu et al. on the SARS-CoV M^{pro} using peptidomimetic aldehydes as electrophilic inhibitors, based on mechanistic similarities [28].

Chandel et al. proposed through virtual screening and *in silico* studies that nelfinavir (Fig. 4b), a recently identified antiretroviral drug used against HIV, can be repurposed for SARS-CoV-2 M^{pro} inhibition [36]. In addition, drugs such as rhein, withanolide-D, withaferin-A, enoxacin and aloe-emodin showed reasonable binding affinity with the enzyme. Similarly, docking studies conducted by Khaerunnisa et al. showed that aside from nelfinavir and lopinavir, the medicinal plant-based natural products kaempferol, quercetin, luteolin-7-glucoside, naringenin and oleuropein displayed promise as potential inhibitors of the SARS-CoV-2 M^{pro} [37].

Aly, using molecular docking, found that aliskiren, a renin inhibitor used to treat hypertension, had better binding affinity for the M^{pro} than did the ligand N3 [38]. It is suggested that potentially reduced expression of ACE2, the host cell receptor of the viral S protein, owing to renin inhibition is likely to be an added advantage of this inhibitor [39]. In addition, the study found that dipyridamole, mopidamol, rosuvastatin and rolitetracycline could be potential anti-COVID-19 agents.

Using molecular dynamics (MD) simulations and docking, Alamri et al. selected three potential inhibitors of SARS-CoV-2 for further studies from an integrated library of 1000 molecules and 16 approved protease inhibitors. Compound 621 (Fig. 5) was the best, although the study found no poses showing thiohemiacetal formation, unlike in previous studies with similar functional groups [40].

Ton et al. combined molecular docking with scaffold optimization of 1.3 billion compounds from the ZINC15 library against the SARS-CoV-2 M^{pro} and identified ZINC000541677852 to have the best binding [41]. Docking studies by Farag et al. found that anthracene anticancer drugs such as daunorubicin and mitoxantrone could be potential inhibitors of the SARS-CoV-2 M^{pro}, but they are unlikely to be useful in treating critically ill COVID-19 patients owing to their adverse side effects [42] (Fig. 5). Interestingly, rosuvastatin was once again found to be one of the most promising candidates during their screening.

Pharmacophore-based virtual screening, followed by molecular docking of the prime leads from the ZINC database, suggested that three compounds, ZINC20291569, ZINC90403206 (Fig. 6a) and ZINC95480156, were ideal candidates to be considered for further studies. All three displayed good binding affinity and non-toxicity, as suggested by ADMET (absorption, distribution, metabolism, excretion and toxicity) analysis [43].

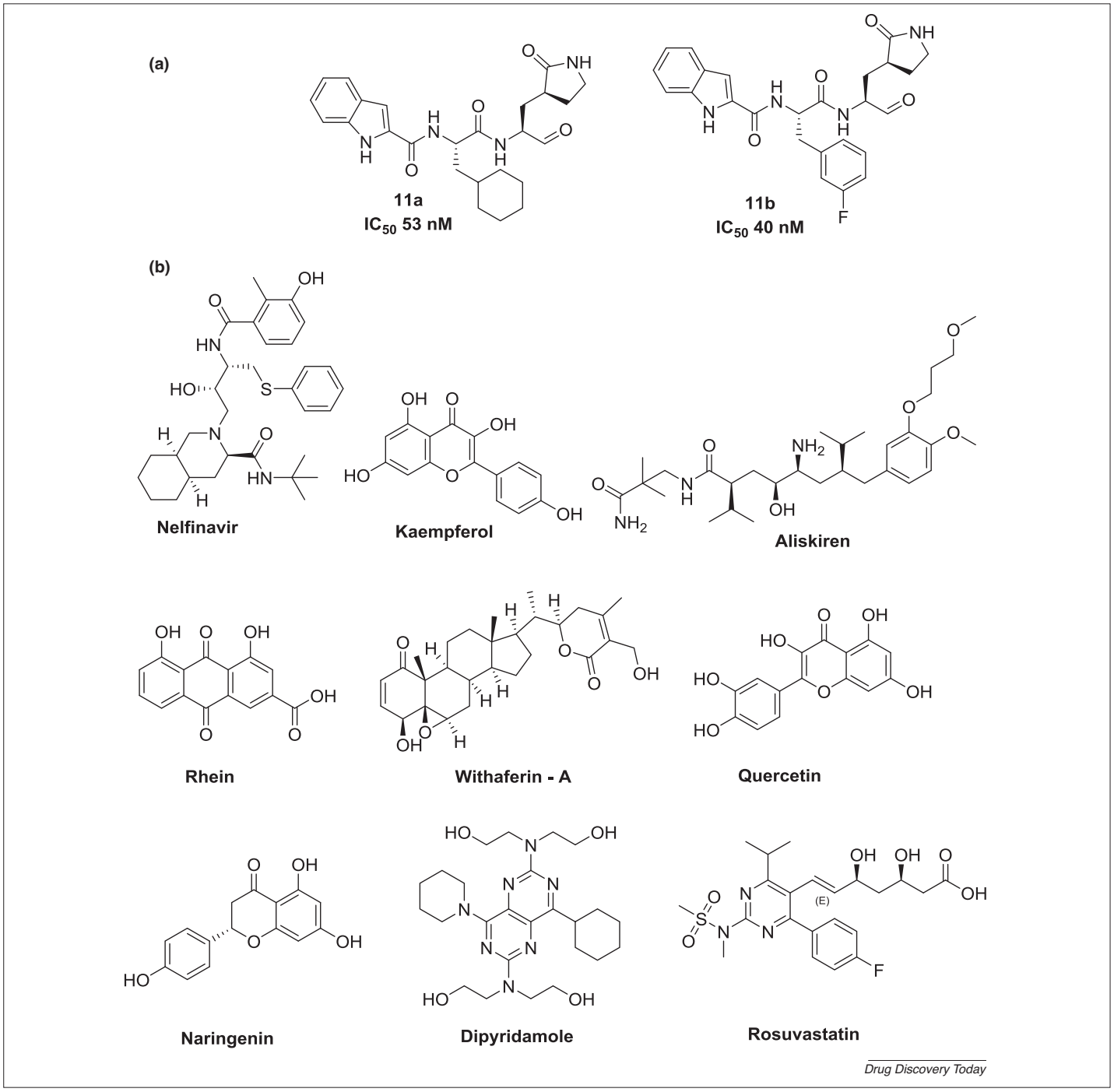

FIGURE 4

(a) Compounds 11a and 11b display excellent antiviral activity [35]. **(b)** Nelfinavir, kaempferol, aliskiren, rhein, withaferin-A, quercetin, naringenin, dipyridamole and rosuvastatin, promising inhibitors of the SARS-CoV-2 M^{pro} [36–38].

A novel virtual screening technique for structure-based ligand design called ligand generative adversarial network (LIGANN) [44] can generate shapes matching the characteristics of the protease-binding pocket. LIGANN followed by lead optimization and molecular docking led to the discovery of compound 27 (Fig. 6a), which displayed the best binding to the enzyme [45].

A virtual screening pipeline followed by ADMET analysis and optimization by comparative docking led to the identification of compounds 12 and 14 (Fig. 6a) as the best inhibitors of the SARS-CoV-2 M^{pro} [46]. The use of artificial intelligence (AI)-based ligand design to generate novel inhibitor leads, followed by lead-based optimization of selected candidates, led to the identification of compounds 46-14-1, 46-14-2 and 46-14-3 as the best candidates for anti-SARS-CoV-2 activity testing [47] (Fig. 6a).

Computational drug repurposing studies using free-energy calculations based on Glide flexible docking followed by molecular-mechanics/Poissson–Boltzmann surface area (MM/PBSA) evaluations identified several neutral drugs, including carfilzomib, eravacycline,

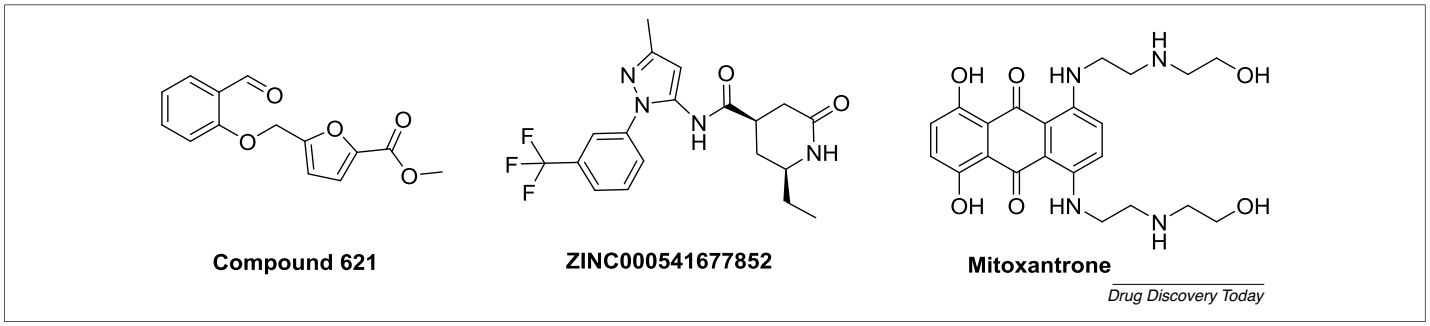

FIGURE 5

Molecular docking of compound 621, ZINC000541677852 and mitoxantrone showed their high binding affinity for the SARS-CoV-2 M^{pro} [40–42].

valrubicin, lopinavir and elbasvir (Fig. 6b) as inhibitors of the SARS-CoV-2 M^{pro}. Carfilzomib had the best binding free energy, whereas streptomycin was a surprising pick among charged candidates [48].

The screening of a library of 32,297 phytochemicals and Chinese medicinal agents with potential antiviral properties against a homology model of the SARS-CoV-2 M^{pro} (derived from the structures of SARS CoV M^{pro}) resulted in 5,7,3′,4′-tetrahydroxy-2′-(3,3-dimethylallyl) isoflavone, myricitrin and methyl rosmarinate (Fig. 6b) being selected as the best candidates [49]. Investigation of the activity of FDA-approved drugs against SARS-CoV-2 M^{pro} yielded sincalide, ritonavir, phytonadione and pentagastrin as possible candidates (Fig. 6b) [50].

In silico screening of bioactive food constituents against the SARS-CoV-2 M^{pro} revealed that phycocyanobilin (Fig. 6c), a chromophore found in cyanobacteria, had better binding affinity than nelfinavir, which has been the subject of many screenings [51]. Also, Adem et al. screened 80 flavonoids and identified hesperidin and rutin, both found in citrus fruits, as having higher binding affinity than nelfinavir [52].

Srivastava et al. found that mepacrine, a derivative of chloroquine, had the best *in silico* results from a list of antimalarial compounds repurposed for the SARS-CoV-2 M^{pro} [53]. Salim et al. screened several compounds isolated from *Nigella sativa* and found that the alkaloid nigellidine and the saponin α-hederin achieved good binding scores [54]. Docking studies conducted on eucalyptol and jensenone (isolated from eucalyptus oil) by Sharma et al. showed potential M^{pro} inhibitory activity [55,56]. Jensenone raises a lot of questions, because theoretical studies alone might not be sufficient to predict the correct mode of binding; the highly electrophilic aromatic aldehydes are expected to have greater reactivity than the peptidomimetic α-ketoamides, with a greater chance for thiohemiacetal formation with Cys145 of the protease.

One study screened a library of 7100 compounds for activity against the SARS-CoV-2 M^{pro}; the compounds included Ayurvedic antitussive molecules used in Indian medicine (the rationale being that coughing is a symptom of the disease), synthetic antivirals and antiviral phytochemicals. Myricitrin, δ-viniferin, taiwanhomoflavone-A, lactucopicrin 15-oxalate, nympholide-A, biorobin and phyllaemblicin-B (Fig. 7a) were selected as the top candidates for M^{pro} binding affinity [57].

Another study conducted by Mishra et al. on nine bioactive compounds from *Anthocephalus cadamba* found that oleanic acid, a substance also found in olive oil, had good binding affinity with the protease [58]. Gentile et al. screened a library of marine natural products, followed by docking and MD simulations on selective compounds, to find heptafuhalol-A, a polyphenol that could be a potential inhibitor with relatively high binding affinity [59].

Based on literature survey of FDA-approved drugs with antiviral and antibacterial properties, Pathak et al. screened ciclesonide, rifampicin, reserpine, loperamide, elvitegravir, brivudine, pentoxifylline, eugenol, isoniazid, tinidazole, diethylcarbamazine and vancomycin using docking studies against the SARS-CoV-2 M^{pro} (Fig. 7b). Rifampicin and ciclesonide yielded the highest binding affinity, and rifampicin was identified as the most promising drug with a good binding energy [60]. The antibacterial and anti-inflammatory effects, respectively, of these two drugs could be added advantages, if they were to be repurposed as protease inhibitors.

Another study based on virtual screening of FDA-approved drugs for repurposing by Kandeel et al. showed that a combination of ribavirin, telbivudine, vitamin B12 and nicotinamide can be used for COVID-19 treatment [61]. Computational synergistic studies by Muralidharan et al. found that a combination of lopinavir, ritonavir and oseltamivir displayed higher binding affinity to the SARS-CoV-2 M^{pro} than each individual drug, which suggests that they can be further explored for repurposing against COVID-19 [62]. However, the antiviral drugs ribavirin and telbivudine are nucleoside analogues, and the likelihood of them being useful as effective protease inhibitors remains low. Conversely, the antiretroviral protease inhibitors ritonavir and lopinavir merit further investigation as inhibitors of the SARS-CoV-2 M^{pro}. It should be noted, however, that a randomized clinical trial of a lopinavir-ritonavir combination treatment involving hospitalized adult patients with confirmed SARS-CoV-2 infection showed no benefit beyond standard care [63].

Another drug-repurposing study for covalent SARS-CoV-2 M^{pro} inhibitors using a combination of molecular docking and a steric clash alleviating receptor (SCAR) screening protocol identified 11 molecules: itacitinib, oberadilol, telcagepant, vidupiprant, pilaralisib, poziotionib, fostamatinib, CL-275838, ziprasidone, leucal/folinic acid and ITX5061. Of these, telcagepant, vidupiprant, poziotinib and fostamatinib (Fig. 7b) were ranked among the best candidates, based on the SCAR protocol [64,65].

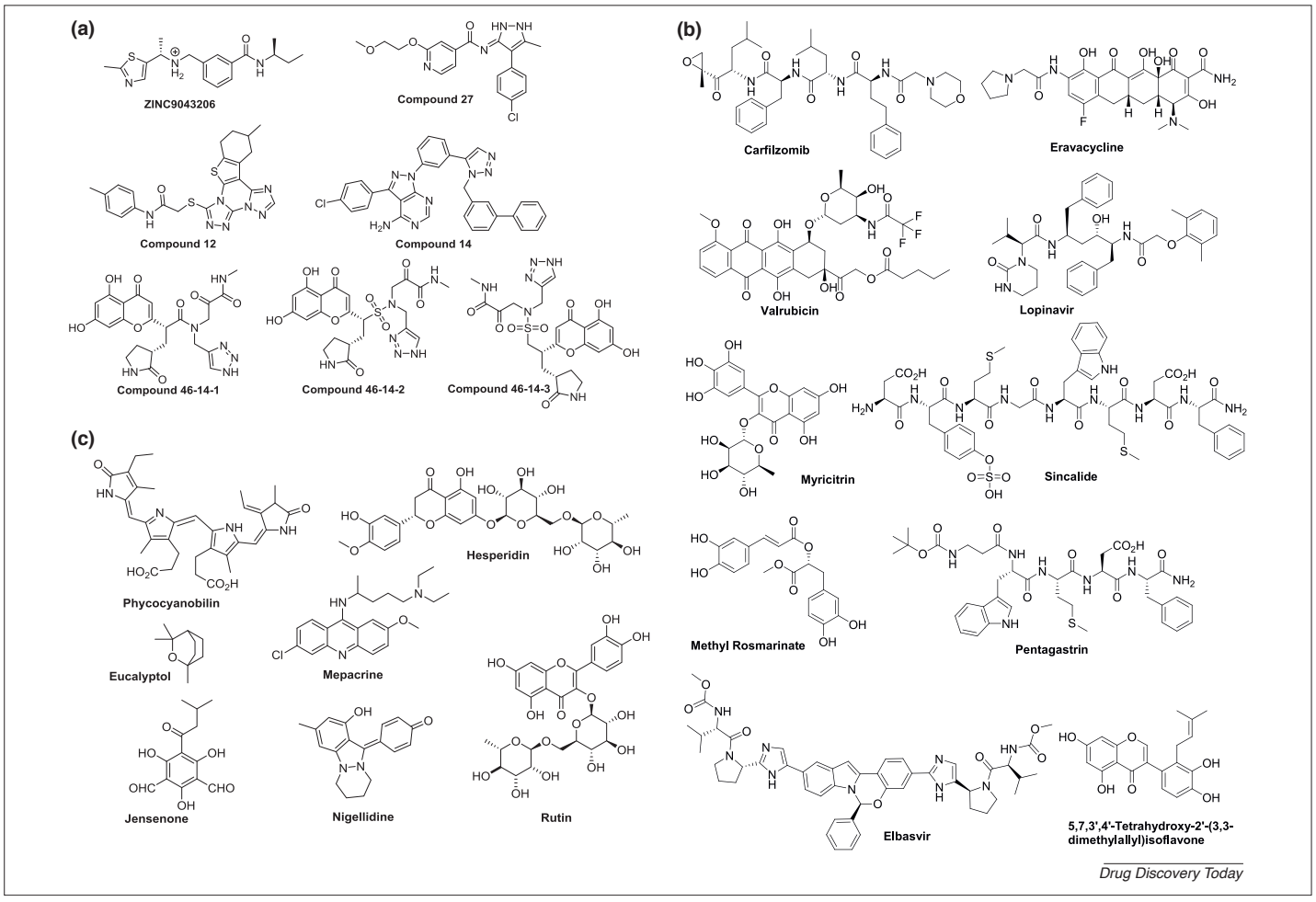

FIGURE 6

(a) Molecules designed by pharmacophore-based virtual screening, LIGANN, comparative docking and AI-based lead optimization for the SARS-CoV-2 M^{pro} [43,45–47]. **(b)** Computational drug repurposing, phytochemical screening and investigation of FDA-approved drugs against the SARS-CoV-2 M^{pro} yielded sincalide, pentagastrin, elbasvir, carfilzomib, eravacycline, valrubicin, lopinavir, myricitrin, methyl rosmarinate and 5,7,3′,4′-tetrahydroxy-2'-(3,3-dimethylallyl) isoflavone as potential inhibitors [48–50]. **(c)** *In silico* screening of several natural products revealed phycocyanobilin, eucalyptol, jensenone, nigellidine, hesperidin and rutin to have potential SARS-CoV-2 M^{pro} inhibitory activity, along with mepacrine, a derivative of chloroquine [51–56].

Gurung et al. [66] screened a library of phytochemicals with previously reported antiviral properties against the SARS-CoV-2 M^{pro} using a computational approach, and they identified bonducellpin-D (Fig. 8) as the best lead molecule. It exhibited higher binding affinity than a list of antiviral drugs, except for nelfinavir, boceprevir and simeprevir and the α-ketoamide [25] used as controls. Gimeno et al. [67] used a combination of three sampling algorithms to select seven possible candidates from a library of approved drugs for SARS-CoV-2 M^{pro} inhibition. Of these, carprofen and celecoxib were selected by the COVID Moonshot initiative for *in vitro* testing. Celecoxib (Fig. 8) was found to inhibit 11.9% of the SARS-CoV-2 M^{pro} at 50 μM and could be a lead molecule for the development of more potent inhibitors, while taking into account the anti-inflammatory as well as the adverse health risks of this drug.

In a search for potential non-covalent inhibitors of SARS-CoV-2 M^{pro}, Zhavoronkov et al. used generative deep-learning approaches to design five novel potential non-inhibitors, INSCoV-181, INSCoV-182, INSCoV-184, INSCoV-185 and INSCoV-188 (Fig. 8) [68], using the inhibitor X77 as a template. Interestingly, the molecules shared peptidomimetic structural patterns and could be considered lead candidates for further structure optimization to develop potent non-covalent inhibitors of the SARS-CoV-2 M^{pro}.

A structure-based drug design approach in combination with immunoinformatics by Panda et al. [69] showed that the non-nucleoside SRN L protein polymerase inhibitor PC786 had better docking scores against the SARS-CoV-2 M^{pro} when compared with the drugs used as controls, such as ribavirin, chloroquine, favipiravir, remdesivir and zanamivir. Only lopinavir had a slightly better docking score. The compound also demonstrated improved binding affinity towards the S glycoprotein and the SARS-CoV-2 RBD–ACE2 complex, and might find a better use in the discovery of vaccine candidates against the virus.

Docking studies of FDA-approved drugs by Verma et al. [70] identified the antimalarial drug lumefantrine and riboflavin at the top of a list of potential SARS-CoV-2 M^{pro} inhibitors. In another study, Strodel et al. [71] subjected more than one million compounds, which included approved drugs, investigational drugs, natural products and synthetic organic

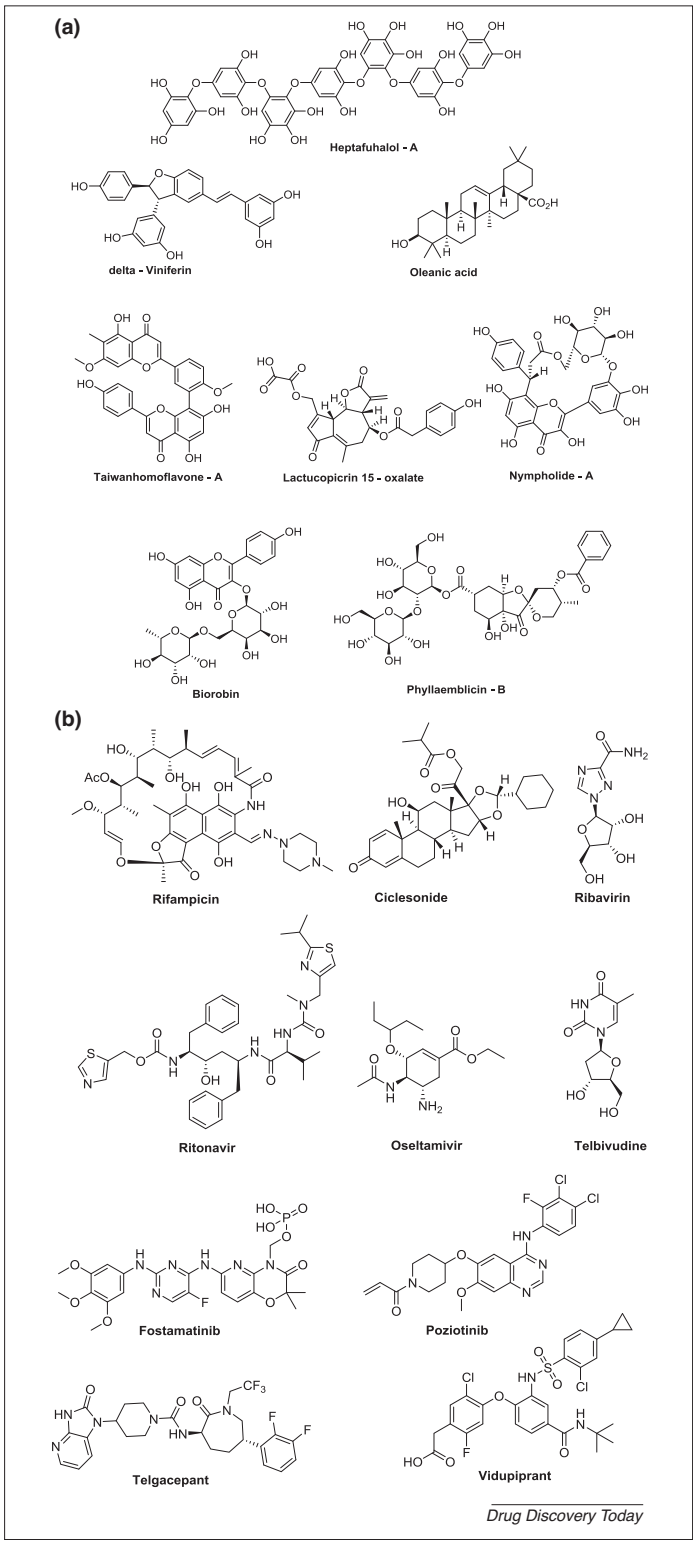

FIGURE 7

(a) Studies conducted on Ayurvedic natural products, plant-based natural products and marine-based natural products gave the best results for myricitrin, δ-viniferin, taiwanhomoflavone-A, lactucopicrin 15-oxalate, nympholide-A, biorobin, phyllaemblicin-B, oleanic acid and heptafuhalol-A [57–59]. **(b)** Screening of repurposed drugs against the SARS-CoV-2 M^{pro} yielded the highest binding affinities for rifampicin, ciclesonide, ribavirin (in combination with vitamin B12, nicotinamide and telbivudine) and oseltamivir (in combination with lopinavir and ritonavir). Telcagepant, vidupiprant, poziotinib and fostamatinib were ranked the best, based on the SCAR protocol for screening covalent ligands against the SARS-CoV-2 M^{pro} [60–62,65].

compounds, to high-throughput virtual screening, and they identified several tyrosine kinase inhibitors and steroid hormones as having high binding affinity to the SARS-CoV-2 M^{pro}. In general, the top binding compounds were characterized by the presence of multiple mono- and bicyclic rings, many of them aromatic, and heterocycles, flexibly linked such that the molecule could adapt to the geometry of the M^{pro} substrate-binding site. Among the natural products, the flavonoid amentoflavone, which has been previously reported to have SARS-CoV-2 M^{pro} inhibitory activity, was found to be the most potent inhibitor of the SARS-CoV-2 M^{pro}.

Using molecular docking, Cheng et al. [72] discovered that alliin (Fig. 8), a bioactive cysteine sulfoxide natural product of garlic, is a putative SARS-CoV-2 M^{pro} inhibitor. Alliin was a more potent inhibitor than remdesivir and ritonavir. Considering its small size, its possession of several heteroatom functionalities that can form hydrogen bonds and its reported biological activities, alliin could be an interesting lead molecule for further development as a SARS-CoV-2 M^{pro} inhibitor, either by itself or when incorporated into hybrid molecules.

Taking into account the flexibility of the active site of the SARS-CoV-2 M^{pro}, Jimenez-Alberto et al. used three conformers of M^{pro} to perform virtual screening of FDA-approved drugs [73] and identified nine molecules as potential inhibitors, including ergoloid, bromocriptine, ergotamine, N-trifluoroacetyladriamycin, amrubicin and daunorubicin. However, the high toxicity of most of these drugs could be an impediment to repurposing them as protease inhibitors.

Bhardwaj et al. used a combination of docking and MD simulations [74] to identify three bioactive compounds, oolonghomobisflavan-A, theasinensin-D and theaflavin-3-O-gallate from the tea plant, as potential SARS-CoV-2 M^{pro} inhibitors, with oolonghomobisflavan being the most promising lead molecule. However, their potential as therapeutically useful SARS-CoV-2 M^{pro} inhibitors could be limited owing to the non-specific binding generally associated with their highly polyphenolic nature.

Using molecular docking and MD simulations, Kumar et al. [75] reported the compounds withanone and caffeic acid phenethyl ester to be better inhibitors of SARS-CoV-2 than withaferin-A, and they were as equipotent as the covalent protease inhibitor N3. Patel et al. [76] showed that fungal metabolites such as bergenin and dihydroartemisinin were slightly better inhibitors than N3 when virtually screened against the SARS-CoV-2 M^{pro}, but not better than the flavonoid glycoside quercitrin. Lyndem et al. [77] screened naturally occurring coumarin derivatives and identified corymbocoumarin, methylgalbanate and heraclenol as potential SARS-CoV-2 M^{pro} inhibitors, but they were less potent than control drugs such as lopinavir or ritonavir.

Ngo et al. [78] screened a database of 4600 compounds found in Vietnamese plants using fast pulling of ligand simulations, and they reported that the naturally occurring compounds cannabisin-A and isoacetoside had better binding affinities for the SARS-CoV-2 M^{pro} than the α-ketoamide inhibitor 13b. A docking and MD simulation study conducted by Ghosh et al. [79] on green tea polyphenols showed that three molecules, epigallocatechin gallate, epicatechin gallate and gallocatechin-3-gallate, had better binding scores than the covalent inhibitor N3 when screened against the SARS-CoV-2 M^{pro}.

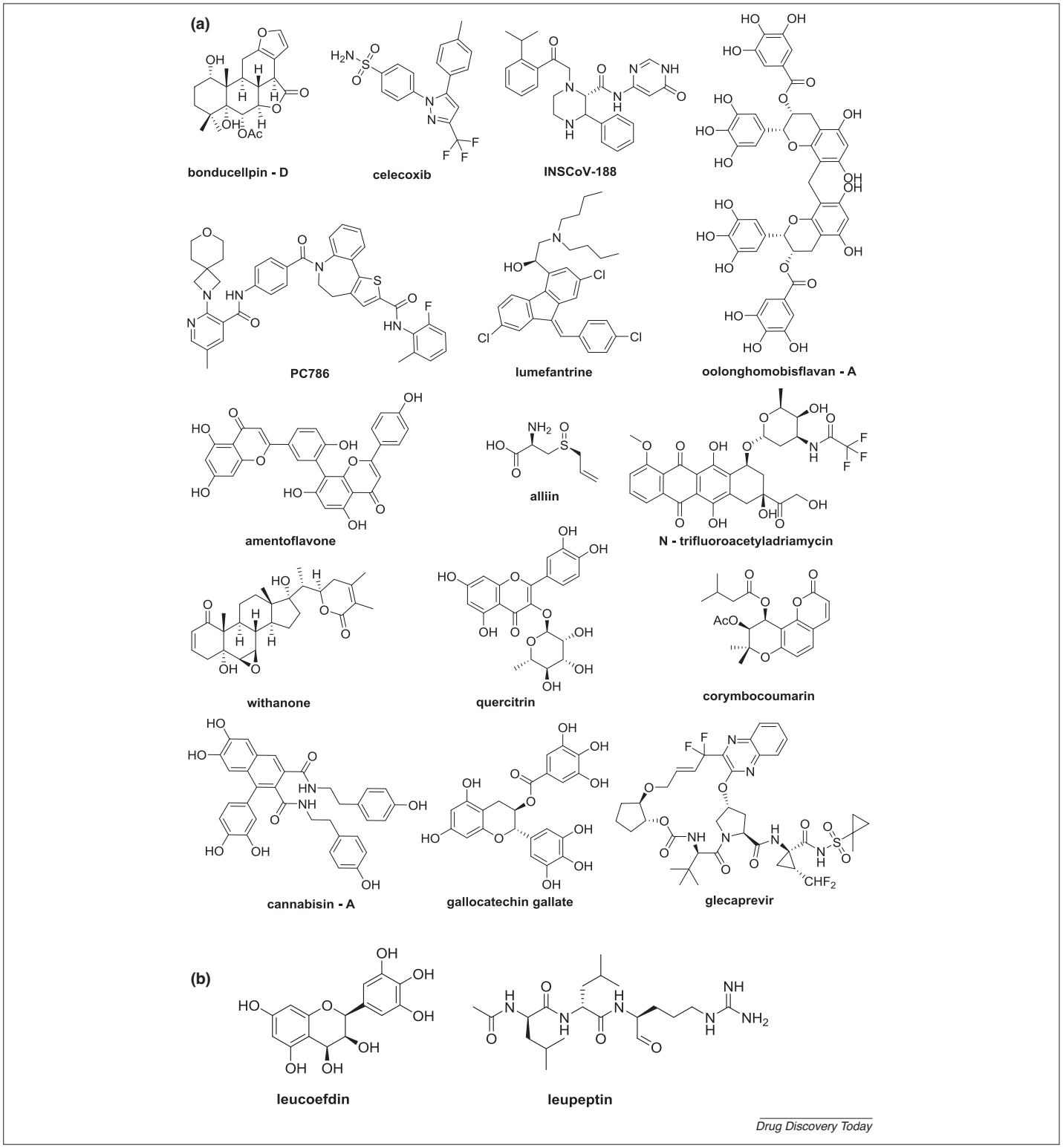

FIGURE 8

Molecules most recently reported to demonstrate potential *in silico* SARS-CoV-2 M^{pro} inhibitory activity [66–83].

Shamsi et al. performed virtual screening of a library of 2388 FDA-approved drugs [80] against the SARS-CoV-2 M^{pro}; from the top ten hits, the antiviral drugs glecaprevir and maraviroc were observed to have the highest binding affinity while satisfying the criterion of binding to the conserved residues in the active site of the M^{pro}. The usefulness of these antiviral drugs for targeting SARS-CoV-2 M^{pro}, either by themselves or in combination with other potential strategies, needs experimental validation and clinical manifestation.

Singh et al. [81] reported that leucoefdin, a molecule found in fruits such as banana and raspberry, has potential as a lead molecule for the discovery of SARS-CoV-2 M^{pro} inhibitors. It displayed a

much better Glide XP docking score than the reference ligand Z31792168 (PDB ID: 5Y84).

Mittal et al. [82] used a structure-guided virtual screening approach based on the covalent inhibitors Michael acceptor N3 and the α-ketoamide 13b; they found that six molecules, leupeptin hemisulphate, pepstatin-A, nelfinavir, birinapant, lypressin and octreotide, formed stable interactions with key conserved residues in the SARS-CoV-2 M^{pro} active site. All the molecules displayed better binding scores than 13b, but none of them scored better than the covalent inhibitor N3. Leupeptin (Fig. 8) is a naturally occurring protease inhibitor. It is peptidomimetic with an aldehyde functionality, which might potentially serve as an electrophilic center for nucleophilic attack by the catalytic cysteine residue of the SARS-CoV-2 M^{pro} active site. It could be a promising candidate for further development as a potential SARS-CoV-2 M^{pro} inhibitor.

Using molecular docking studies, Das et al. [83] showed that rutin, a molecule already identified in a previous study, had the highest inhibitory efficiency from a group of 33 molecules screened. It had better binding scores than the reference drugs lopinavir and ritonavir.

Conclusion

The development of SARS-CoV-2 M^{pro} inhibitors is in its fledgling stage, and the urgency of the situation has prompted a rush towards the repurposing of previously approved drugs. The high level of structural conservation among the M^{pro}s of 12 different CoVs can be exploited to design pan-inhibitors of viral proteases, and target-specific inhibitors could be developed for the SARS-CoV-2 M^{pro}.

The crystal structure of the SARS-CoV-2 M^{pro} complexed with an irreversible inhibitor was first reported in early February 2020. It showed Michael addition by Cys145 to inhibitor N3, resulting in irreversible covalent inhibition, as was observed with the SARS-CoV M^{pro} and inhibitor N1. This, along with alternative mechanistic insights gained from the rational design of peptidomimetic aldehydes, serves to lay down some ground rules for the development of covalent inhibitors of the M^{pro}. Because covalent inhibitors in general can be promiscuous and can cause off-target effects, care should be exercised in designing such covalent inhibitors to be strictly target-specific.

An important parameter to be considered in designing such inhibitors is the drug-target residence time, which is dependent on the rate constant for dissociation of the inhibitor–target complex [84–86]. Of the covalent inhibitors, the high rate of inactivation of the enzyme by N3 suggests a low dissociation constant and rapid covalent inactivation by the Michael acceptor, and it might prove useful in mitigating the drug side effects. Conversely, α-ketoamides such as 11 r and 13b have the advantage that, when compared with Michael acceptors, the thiohemiacetal intermediate formed is stabilized by an additional hydrogen bond with the catalytic center of the protease. This, along with the reversibility of the nucleophilic addition to carbonyl groups, will be advantageous in avoiding off-target effects. There are already α-ketoamide protease inhibitors in clinical use for hepatitis C viral infection. Both of these classes of molecules are useful leads for developing covalent inhibitors of the SARS-CoV-2 M^{pro}.

Peptidomimetic aldehydes are also capable of forming thiohemiacetal intermediates that act as transition state analogues, although they can be metabolically less stable. They, too, have the potential of being developed as SARS-CoV-2 M^{pro} inhibitors. The incorporation of small-molecule covalent inhibitors such as ebselen into hybrid molecules might provide additional leads for covalent inhibitors.

X-ray crystal structures and molecular docking have identified some of the key non-covalent interactions that guide the binding of inhibitor molecules inside the M^{pro} substrate-binding pocket. Such information gathered through the structure analysis of a wide array of reported non-covalent inhibitors with diverse structural elements should be the basis for designing and developing new inhibitors. A factor that should be taken into account in designing such inhibitors, besides the binding affinity, is the ability of the molecules to establish interactions with the enzyme residues that have been recognized to stabilize ligands in the active site. Among the wide range of natural products that have been identified as potential SARS-CoV-2 M^{pro} inhibitors are a large number of flavonoids, the flavonoid glycosides being the most active of them. Flavonoids in general exhibit a range of biological activity, which could be attributed to their polyphenolic character and ability to form multiple hydrogen bonds. Although they might not prove useful as specific SARS-CoV-2 M^{pro} inhibitors by themselves, they could be used as templates for designing new inhibitors.

Additionally, small-molecule bioactive natural products such as alliin could be a useful source of leads for structure optimization and incorporation into hybrid molecules as SARS-CoV-2 M^{pro} inhibitors. An inhibitor that selectively targets the M^{pro} can be an effective first line of defense against COVID-19, and CoVs in general, either as a single agent or in combination with other antiviral therapies.

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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