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Current Opinion in Virology

Virus structure and structure-based antivirals

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Structure-based antiviral developments in the past two years have been dominated by the structure determination and inhibition of SARS-CoV-2 proteins and new lead molecules for picornaviruses. The SARS-CoV-2 spike protein has been targeted successfully with antibodies, nanobodies, and receptor protein mimics effectively blocking receptor binding or fusion. The two most promising non-structural proteins sharing strong structural and functional conservation across virus families are the main protease and the RNA-dependent RNA polymerase, for which design and reuse of broad range inhibitors already approved for use has been an attractive avenue. For picornaviruses, the increasing recognition of the transient expansion of the capsid as a critical transition towards RNA release has been targeted through a newly identified, apparently widely conserved, druggable, interprotomer pocket preventing viral entry. We summarize some of the key papers in these areas and ponder the practical uses and contributions of molecular modeling alongside empirical structure determination.

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Introduction

Since the beginning of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic in 2019, there has been a race between antiviral drug design and vaccine development to combat the disease. Although vaccines won the race, lowering the risk of severe infection, there is still a need for antiviral drugs, driving research for SARS-CoV-2, and other significant viral pathogens. The literature in the past two years has been dominated by studies utilizing computational methods to identify SARS-CoV-2 antivirals [1–15]. In this Opinion we focus on SARS-CoV-2 and picornaviruses, and antiviral leads where structural data have contributed significantly to the design or interpretation of the antiviral mechanism and led to insightful information for future structure-based drug design.

SARS-CoV-2

SARS-CoV-2 belongs to the *Coronaviridae* family, a family of pleomorphic ss(+)RNA viruses. Its genome is associated with the N protein forming the nucleocapsid, coated with a spike glycoprotein-adorned membrane [16]. The most promising drug targets against SARS-CoV-2 have been the spike glycoprotein (S) and the non-structural components: the main protease (M^{pro}), the papain like protease (PL^{pro}), and the RNA-dependent RNA polymerase (RdRp) [7,17–23]. For the non-structural, highly conserved proteins, drug repurposing and broad range inhibition was an attractive approach [3,11,18–22,24–28,29•,30••,31–45]. S, as a surface protein is antigenic and is prone to mutations [17]. A complementary strategy of inhibiting host proteins required in infection is exemplified by the use of soluble peptides mimicking the angiotensin-converting enzyme (ACE2) receptor binding domain (RBD) [46,47].

Picornaviruses

The noticeable structural and functional conservation between the proteases and polymerases of SARS-CoV-2 and those of picornaviruses has led to research focusing on broad-range inhibitors targeting both families. Picornaviruses are a family of small, 30 nm icosahedrally symmetric viruses with (+)ssRNA genome. The capsids consist of three β-barrel viral proteins (VP1, VP2, and VP3) and one elongated internal protein, VP4. Many of the viruses in the family have a conserved hydrophobic pocket in VP1 that can contain a lipid factor [48,49]. Several picornavirus inhibitors target this conserved pocket, preventing genome release, but none of the inhibitors are currently in clinical use [50]. We will describe advances targeting a novel interprotomer capsid pocket instead.

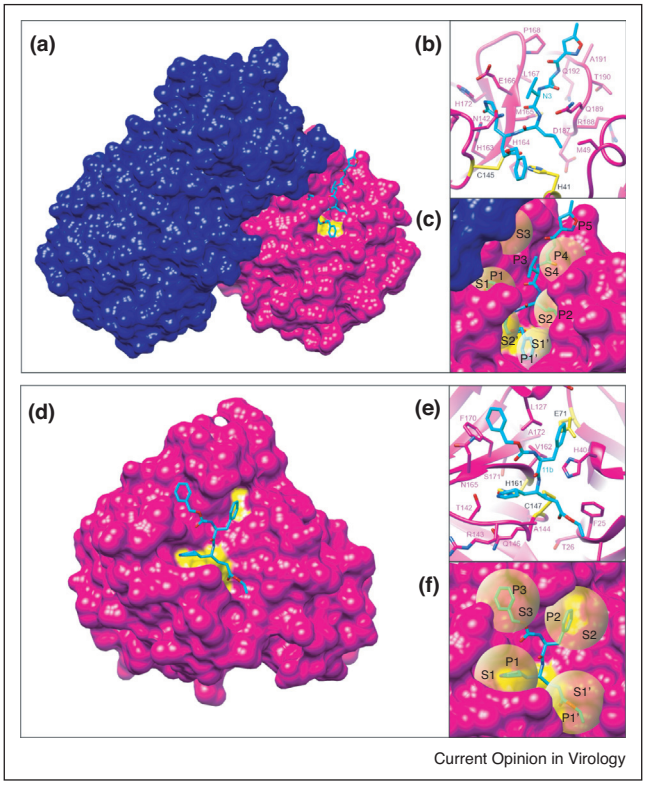
Drug-repurposing and computational structure-based drug design

Drug-repurposing has rapidly advanced SARS-CoV-2 antiviral approaches avoiding lengthy clinical drug approval processes, building on work done to inhibit SARS-CoV, MERS-CoV, hepatitis C and so on. Amongst the drugs which have shown the most promising results and have gone on to clinical trials are the hepatitis C antivirals, telaprevir, narlaprevir and boceprevir inhibiting M^{pro} and remdesivir, ribavirin, suramin and favipiravir riboside triphosphate (RTP) against the RdRp [31–34,44,51]. SARS-CoV-2 M^{pro} was a very promising target at the start of the pandemic and its potential and the advances made have been extensively reviewed: Mengist *et al.* reviewed their toxicology and the structural basis for their inhibition while some other reviews have focused on their potential for clinical use and future prospects [18–22,39,40]. Like many other non-structural proteins, M^{pro} is highly conserved in structure and substrate specificity across the *Coronaviridae* family as well as some other virus families, like the *Picornaviridae*. Both M^{pro} and the picornaviral $3C^{pro}$ have chymotrypsin-like folds and cleave the viral polyprotein into functional proteins, which in turn are involved in viral replication. SARS-CoV-2 M^{pro} is a homodimer with a Cys-His catalytic dyad between two domains (Figure 1a). M^{pro} compared to $3C^{pro}$ has an additional α-helical domain connected by a loop to the catalytic site. Its substrate-binding pocket subsites and the corresponding inhibitor positions are shown in Figure 1c. Unlike SARS-CoV-2 M^{pro}, $3C^{pro}$ is a monomer and has a Cys-His-Glu/Asp catalytic triad (Figure 1d). Both proteases require Gln as the P1 residue in the substrate and only have space for a small amino-acid residue at P1′ (Figure 1) [30••,35,36]. For this reason, inhibitors targeting SARS-CoV M^{pro} and picornavirus $3C^{pro}$ were used as templates for design of SARS-CoV-2 M^{pro} [24,26,30••,35–38,41,52,53].

Over 1000 M^{pro} structures have now been deposited in the PDB giving an unprecedented opportunity for detailed data mining. Although M^{pro} is catalytically active as a homodimer, almost all modeling studies have been based on the monomeric structure. Weng *et al.* (2021), however, utilized and validated the effect of dimers for inhibitor binding on the molecular level [54]. The initial excitement over repurposing of protease inhibitors has so far not led to effective clinical use and this may well be partially due to the little knowledge on how dimerization affects the inhibitor binding region.

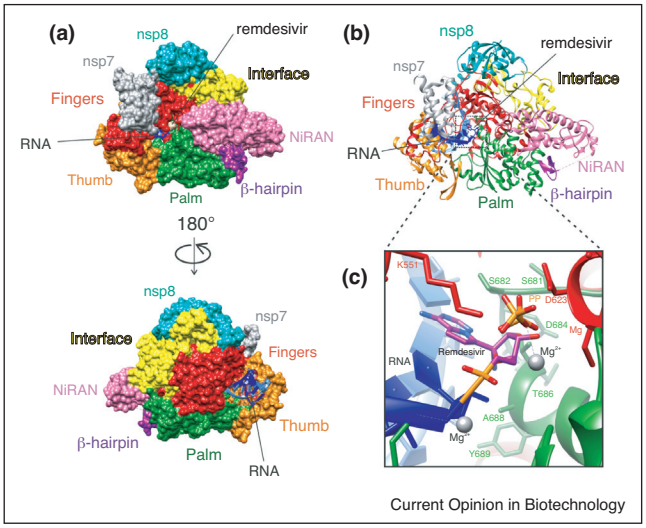
The RdRp has also been explored as a target for drug repurposing [18,20,27,29•,43,45]. It is a vital enzyme transcribing RNA from its complementary template. Structurally, it resembles other right-hand polymerases consisting of 3 conserved domains termed the palm, fingers and thumb (Figure 2). The structural similarity of the RdRp across families of RNA viruses was recently used by Mönttinen *et al.* to create a phylogenetic tree. SARS-CoV-2 RdRp clusters with several flaviviruses and hepaciviruses, adding weight to repurposing the previously mentioned inhibitors remdesivir, suramin and ribavirin, all developed as hepatitis C antivirals [25]. Structural characterization of the SARS-CoV-2 RdRp started with the description of the complex with accessory factors nsp7 and nsp8 [27,28]. Complexes with the RNA template and finally with the nucleotide-analog remdesivir (Figure 2) progressively improved the understanding of RdRp activity and the mechanism of nucleotide-based

Figure 1



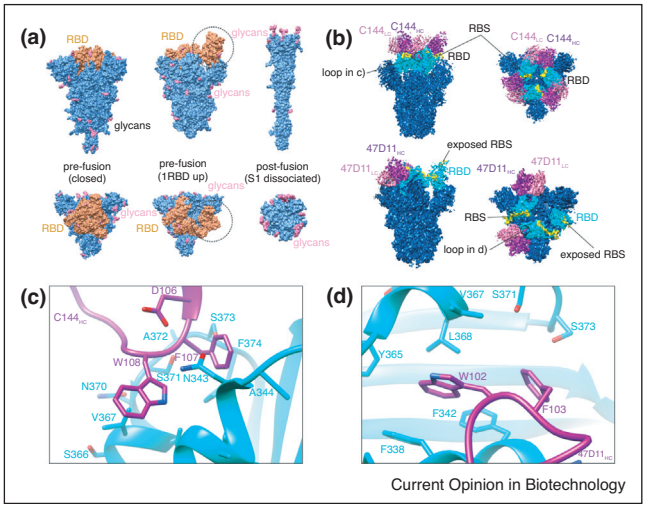
Structure of SARS-CoV-2 M^{pro} and coxsackievirus B3 $3C^{pro}$ bound with inhibitors.
(a) Surface representation of SARS-CoV-2 M^{pro} (PDB: 6LU7) with monomers in blue and magenta. Catalytic diad is marked in yellow and inhibitor in cyan. **(b)** Close up of the active site residues and interactions with the inhibitor N3 [30••]. **(c)** Close up of the active site and the inhibitor N3 showing active site subsites with yellow circles and inhibitor positions marked P1′-P5 [30••]. **(d)** Surface representation of coxsackievirus B3 $3C^{pro}$ in magenta with inhibitor 11a in cyan and catalytic triad in yellow (PDB: 3ZYD) [24]. **(e)** Close up of the active site residues and interactions with inhibitor 11a. **(f)** Close up of the active sites and the inhibitor 11a showing active site subsites with yellow circles and inhibitor positions marked P1′-P3 [24].
Figure created in UCSF Chimera [87]. (M^{pro} = main protease, $3C^{pro}$ = 3C protease).

Figure 2



SARS-CoV-2 RdRp with nsp7, nsp8, RNA template and remdesivir. **(a)** Surface and **(b)** ribbon view of RdRp structure with color-coded domains labelled. (b) Remdesivir can be seen in the active site. **(c)** Close up view of remdesivir and its location in the RdRp. Pyrophosphate (PP) is marked in orange and is a byproduct of the remdesivir-inhibited reaction [34]. Figures created in UCSF Chimera [87] from PDB:7BV2. Nidovirus RdRp-associated nucleotidyl transferase domain (NiRAN).

Figure 3



SARS-CoV-2 spike glycoprotein conformational states, glycosylation and binding of C144 and 47D11 Fab.
(a) Side and top view of a surface representation of the spike glycoprotein in pre-fusion closed (PDB ID: 6XR8), pre-fusion open (PDB ID: 6XM4) and post-fusion conformations (PDB ID: 6XRA) [58,88]. RBDs are shown in orange and glycans in pink. **(b)** Side and top views of the cryo-EM density maps showing C144 FAb complexed to closed pre-fusion S (top; EMD-22737) and 47D11 FAb complexed to pre-fusion S with 1 RBD up (bottom; EMD-11812) [66••,67•]. RBD in cyan and RBS in yellow. One RBS in the S protein bound with 47D11 is exposed and accessible to ACE2. Black circle in the C144-S1 complex marks the position of the loop shown in **(c)**. Black circle in the 47D11-S complex marks the position of the loop shown in **(d)**. (c) Enlarged view of the residues involved in the hydrophobic pocket on S protein RBD (cyan) and $C144_{HC}$ CDRH3 loop (purple) (PDB ID: 7K90) [66••]. (d) Enlarged view of the residues involved in the hydrophobic pocket on S protein RBD (cyan) and $47D11_{HC}$ CDRH3 loop (purple) (PDB ID: 7AKD) [67•]. Figures were made in UCSF Chimera [87].

inhibitors [27,34]. A cryo-EM structure with suramin further indicates the potential for distinct chemical entities against SARS-CoV-2 RdRp [44]. The structural conservation between SARS-CoV-2 and flavivirus RdRp indicates a potential alternative option for drug discovery: two inhibitors targeting the flexible RNA tunnel have been described for Dengue virus RdRp. The interacting residues are largely conserved across flaviviruses and may allow the design of broad-spectrum antivirals along with those targeting the active site [55,56]. While the lack of flavivirus RdRp structures in their catalytically competent state represents a challenge for structure-based drug discovery approaches, a recent structure of TBEV RdRp helped to identify the differences in catalytic sites between tick-borne and mosquito-borne flaviviruses. These differences were shown to be vital in host recognition, expanding the known RdRp roles in the infection cycle [45,57].

Structural proteins as targets

Viral structural proteins are the first to interact with the host whether by interacting with other proteins such as receptors or antibodies, or the host environment in the form of pH, ions or lipids. As such, they are prone to mutations to evade the host immune system. In SARS-CoV-2 infection, S protein attaches to the host receptor, ACE2, and fuses the viral and cell membranes. The S trimer (Figure 3), has monomers composed of S1 and S2 domains which are cleaved by furin making the spike fusion-active. The receptor binding domain (RBD) on S1 can be either in a 'down' conformation hiding the receptor binding site (RBS) or in an 'up' conformation exposing the RBS (Figure 3a). Upon binding to ACE2, S2 undergoes conformational changes exposing the fusion peptide, and S1 dissociates leaving the post-fusion conformational state (Figure 3a) [58,59]. ACE2 glycosylation was shown to be important in its affinity for S [62]. Co-receptors include transmembrane protease serine 2 (TMPRSS2), and neuropilin binding the cleaved S [60,61].

All newly emerging SARS-CoV-2 variants of concern have amino acids changes that are being monitored in the S protein RBD (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/ accessed 10th August

2021). *In vitro* evolution driven to increase S protein's affinity to ACE2 managed to recreate the emergence of the new variants of concern, Alpha, Beta and Gamma [47]. The authors showed that the ACE2 RBD domain was a highly potent inhibitor competing with membrane-bound host ACE2 for both existing strains and the newly predicted ones, confirmed by cryo-EM of existing strains [47].

One approach for inhibiting the S protein is by neutralizing antibodies (NAb), but useful epitopes must take into account the heavy surface glycosylation of S (Figure 3a). Sikora *et al.* mapped the spike protein glycans looking for unshielded epitopes using molecular simulations. This resulted in a much larger covered area than previously thought and suggests that the spike stalk is not a favorable antibody target [63]. Casalino *et al.* have done a similar mapping using all-atom molecular dynamics, finding the importance of two glycans shielding the spike RBD. The glycans play a role in changing the spike protein from the closed to the open state, as after their deletion, most spikes remained in the closed state, unable to bind to ACE2 [64•]. Hence spike production for vaccination or other functional studies on epitopes needs to be done in mammalian cells for correct glycosylation [65].

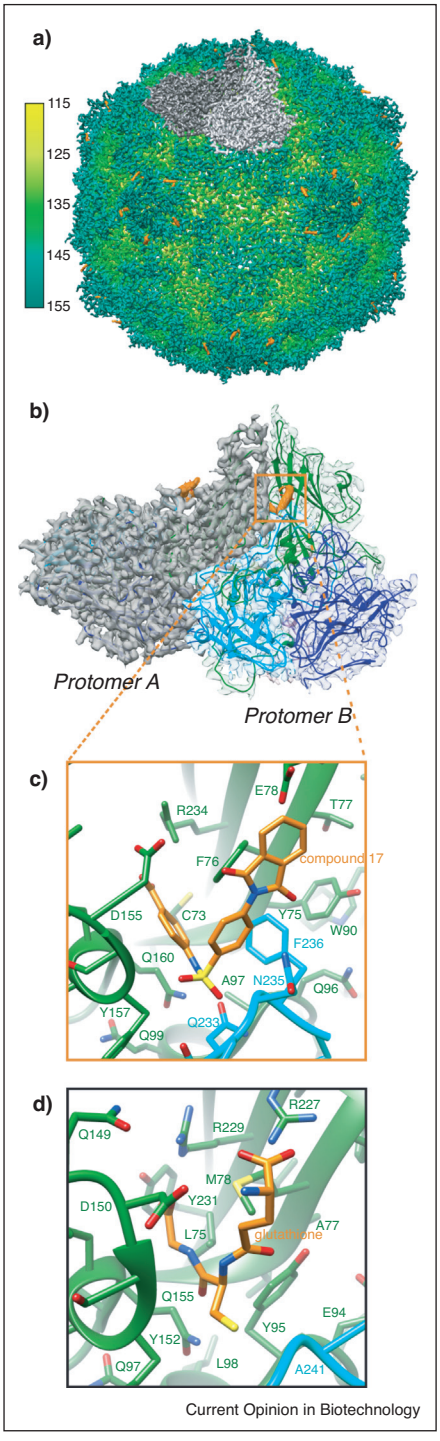
Based on the epitope location, NAbs' mode of action can be competition with ACE2 for the RBS on the RBD, stabilization of the spike by binding distally to the RBS either in an 'up' or 'down' conformation of the RBD (Figure 3), and destruction of the spike by driving spike conformation to post-fusion [66••,67•,68–70]. Barnes *et al.* have tested and classified Covid19 patients isolated NAbs which stabilize the spike or compete with ACE2 in three classes based on the binding site [66••]. With the emergence of new variants, a need for an antibody binding distally from the RBS arose [67•]. One of the earliest antibodies (CR3022) tested against SARS-CoV-2 was shown to potently bind the RBD at an epitope which is only exposed in the open conformation. Interestingly, using cryo-EM, no density was observed for the CR3022 FAb on the 'one up — two down' S trimer conformation which should have one epitope exposed. Instead, increasing the sample time after antibody addition yielded a homogenous population of post-fusion S proteins, suggesting that CR3022 drives S1 dissociation [69]. A similar mode of action has been observed for some Ebola antibodies, where, as with CR3022, infection is partially inhibited [69,71]. Together these findings indicate that antibodies binding to an epitope away from the RBS, stabilizing the pre-fusion closed S trimer, should be the most effective (Figure 3). Successful vaccine production is based on a similar principle, the S trimers are stabilized in a pre-fusion conformation by substitution with double proline and removal of the furin cleavage site [72]. Recent advances have been made on exploiting S proteins stabilized in the closed conformation to produce antibodies which bind to epitopes distant from the RBS [73–76]. One such antibody is 47D11 stabilizing the S trimer in a 'one up — two down' conformation leaving one RBS accessible to ACE2 (Figure 3b down, d). It binds in a conserved hydrophobic pocket that is usually covered by glycan N343 (Figure 3d). It stabilizes the glycan in an upright position and inserts a CDRH3 loop in the pocket. Its activity was also verified against newly emerging SARS-CoV-2 variants [67•]. The C144 antibody has an epitope which is accessible in both 'up' and 'down' conformations and upon binding it spans across adjacent RBS and stabilizes the trimer in the closed conformation (Figure 3b up). It contains the same loop as 47D11, interacting with the hydrophobic pocket (Figure 3c). Its binding site still partially overlaps with the RBS (Figure 3b up) and it is not effective against all variants indicating that this approach should be explored further [66••].

Nanobodies and aptamers offer alternative, potentially cheaper, easier, faster approaches to NAb. Nanobodies are variable regions (VHH) of antibodies expressed alone. Huo *et al.* and Xiang *et al.* have shown the potential of nanobodies in inhibiting SARS-CoV-2 attachment to the host cell [77,78]. By screening through the VHH library against purified RBD Huo *et al.* discovered 2 nanobodies H11-H4 and H11-D4 with binding sites which partially overlap with the RBS. H11-H4 and H11-D4 both bind to the RBD in 'up' and 'down' conformations, stabilizing the S protein in 'one up — two down' state as shown by isothermal titration calorimetry (ITC) and X-ray crystallography [77]. Xiang *et al.* noticed this effect was increased when using multivalent nanobodies [78]. Koenig *et al.* produced multivalent nanobodies that when bound resulted in an irreversible, fully open conformation preventing S binding to the receptor. What is more, they have tested the activity in evolutionary mutants and discovered that a homotrivalent nanobody was not effective after a single mutation, however a multivalent nanobody targeting different epitopes significantly hindered the emergence of mutants [79••]. More recently, synthetic nanobodies competing with ACE2 have shown their effectivity in hamsters [80].

Aptamers have shown potential to compete with ACE2 as nasally administered drugs [81]. For Nab, nanobodies and aptamers, the neutralization efficacy can be computationally predicted, measured through binding assays like ELISA, structural studies, pseudovirus or virus neutralization in cell culture, before going on to animal trials. Experience over the past two years has emphasized that even efficacy in ELISA does not necessarily correlate to infection inhibition in cell culture [68].

One of the central themes in inhibition of virus entry is to prevent conformational changes required to release the

Figure 4



Picornavirus' newly identified conserved site bound with inhibitor 17 bound to CVB3 and glutathione to EV-F3.
(a) Electron density map of CVB3 at 2.8 Å-resolution bound with an inhibitor (compound 17) radially colored according to the key in Å, viewed down a two-fold axis of symmetry. The position of the inhibitor is shown in orange. Two protomers are shown in light and dark grey [83••]. **(b)** Detailed view of the interprotomer position of compound 17 in CVB3, with one protomer shown in dark grey and the second protomer in light grey surface with the atomic models of VP1 (green), VP2 (blue) and VP3 (cyan) shown in ribbon [83••]. **(c)** Close up view of compound 17 and interactions with the CVB3 site residues (PDB: 6ZCL) [83••]. **(d)** Close up view of a similar site in enterovirus-F3 as in (c) with glutathione in the position corresponding to compound 17 in CVB3 (PDB: 6T4C) [86]. (a) and (b) are adapted and reproduced with permission from Flatt *et al.* [83••], both under a Creative Commons Attribution 4.0 International License. (c) and (d) were made with UCSF Chimera [87].

genome such as the efforts to prevent SARS-CoV-2 S fusing with the host membrane described above. In picornaviruses, it has long been recognized that expansion of the capsid and exposure of VP1 and VP4 termini help in releasing the genome into the cytoplasm triggered by albumin, ions, receptor binding and/or pH. Long-term efforts to stabilize the picornaviral capsids by binding molecules to the VP1 hydrophobic pocket have recently been complemented by the description of another druggable pocket conserved in enteroviruses (Figure 4). The interprotomer pocket 16 Å away from the VP1 hydrophobic pocket, can accommodate small molecules, which stabilize the capsid in viruses belonging to Enterovirus A [82,83••,84,85]. A series of compounds were active in cell culture preventing virus infection, some of the residues involved in binding are conserved, and essential, others could be mutated, but with a subsequent reduction in viral viability. Initial studies with CVB3 have recently been taken to higher resolution, including a new atomic model with a broader range inhibitor bound to CVB4 [82,83••]. Duyvestyen et al. showed that glutathione, previously identified as a common cellular factor involved in enterovirus capsid assembly, binds to a similar site on enterovirus F3 (Figure 4d) [86].

Conclusions

Antivirals targeting vital non-structural viral proteins prevent replication or polyprotein maturation. As their targeted conserved sites are different from the host's, any antivirals targeting them should have reduced chances for off-target activity. Drug repurposing and design of broad range inhibitors are attractive approaches for finding antivirals against such targets. This has yielded a list of antivirals with partial efficacy across many targets, and sped up the search for SARS-CoV-2 inhibitors tremendously. In the case of remdesivir, it provided a quick remedy for the most critical patients in their recovery very early in the pandemic. The structural proteins, as they are exposed to the immune system, are prone to mutation. Therefore, specific inhibitors, rather than broad range inhibitors, are more common. Antivirals targeting structural proteins target the receptor attachment, membrane fusion and/or genome release, thus stopping infection at an early stage. Here, most success has been seen with antibodies, especially through the successful COVID-19 vaccine campaign targeting S. This strategy necessitates constant updating of the diagnostics, antivirals and

vaccines to ensure that variants of concern are susceptible to these proven measures. In the long run, as long as any particular virus is widespread in the population, new variants will continue to emerge, some of which will be resistant. Therefore, a combined approach that utilizes both preventative and therapeutic measures will be of benefit. To search for antivirals, virtual screening and other modeling methods have been heavily exploited. Unfortunately, most of the published modeling-oriented studies lack wet lab experimental validation. This is, in our opinion, insufficient, since any molecular modeling method alone cannot reliably predict biological activity. Commonly, molecular dynamics simulations are too short (they should be on the microsecond scale) and are carried out with too simplistic systems, for example, with many proteases, such as M^{pro}, monomers have been used in the majority of studies, while dimers are biologically relevant. Whereas structural methods and binding affinity assays support our understanding of the mechanisms behind biological activity assays in cell culture and animal models, alone they are insufficient to infer inhibitor efficacy. One cannot forget the importance of host factors in the virus infection cycle and viral surface protein glycosylation, metastability and dissociation when designing antivirals. All of these factors may contribute to the lack of efficient antivirals so far in clinical use. The methods and models, for example, organoids used for screening, need improvement, alternative targets, especially targeting host factors that are not so prone to mutation, need to be explored more systematically, and continual monitoring of circulating virus variation should all contribute to the field in the near future.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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