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The impact of high-resolution structural data on stemming the COVID-19 pandemic

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The coronavirus disease 2019 (COVID-19) pandemic has had a catastrophic impact on human health and the world economy. The response of the scientific community was unparalleled, and a combined global effort has resulted in the creation of vaccines in a shorter time frame than previously unimaginable. Reflecting this concerted effort, the structural analysis of the etiological agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has progressed with an unprecedented pace. Since the onset of the pandemic, over 1000 high-resolution structures of a broad range of SARS-CoV-2 proteins have been solved and made publicly available. These structures have aided in the identification of numerous potential druggable targets and have contributed to the design of different vaccine candidates. This opinion article will discuss the impact of high-resolution structures in understanding SARS-CoV-2 biology and explore their role in the development of vaccines and antivirals.

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Introduction
The coronavirus disease 2019 (COVID-19) pandemic has resulted in a global crisis with devastating effects on public health and the global economy. The scientific community has invested tremendous efforts into characterizing and understanding severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of COVID-19. An unprecedented volume of data has been produced, providing the scientific world with a plethora of information on SARS-CoV-2 biology. Shortly after publishing the SARS-CoV-2 genome sequence (Figure 1a) the first high resolution structures emerged, allowing for the rapid identification of potential targets for therapeutic intervention. These structures contributed to a molecular understanding of the mechanisms of fundamental processes of the SARS-CoV-2 life cycle, such as virion attachment, entry, transcription/genome replication, assembly and egress, and provide a foundation for the targeted development of effective strategies to combat viral infection.

SARS-CoV-2 is a positive strand RNA virus in the betacoronavirus subfamily, which also includes the original SARS-CoV and middle east respiratory syndrome coronavirus (MERS-CoV). The SARS-CoV-2 genome comprises approximately 30 kb of RNA that encodes for 29 viral proteins (Figure 1b). Using a combination of X-ray crystallography, cryo-electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR), over 1000 structures of 18 different SARS-CoV-2 proteins have been deposited in the protein data bank in the timeframe of February 2020 to March 2021 (Figure 1c). Numerous druggable targets for inhibition of SARS-CoV-2 have been proposed based on these structures. This opinion article will focus on those most promising for pharmacological intervention: the main viral protease (Nsp5/Mpro/3-CLpro), the SARS-CoV-2 RNA-dependent RNA polymerase (Nsp12/RdRP), and the viral spike (S) protein.

The SARS-CoV-2 main protease (nsp5/Mpro/3CLpro)
Viral proteases have been successfully targeted to treat other viral infections, such as those caused by human immunodeficiency virus (HIV) and hepatitis C virus (HCV). The SARS-CoV-2 main protease (Mpro) is an essential cysteine protease that is required for cleaving the viral precursor polyproteins, including all of the precursors of the SARS-CoV-2 replication/transcription complex (RTC) (Figures 1b and 2 a-b) [1]. Mpro is highly conserved between SARS-CoV-2 and other betacoronaviruses such as SARS-CoV (96% sequence similarity) [2]. Compared to host serine proteases, however, it differs in substrate selectivity, preferring a glutamine residue in P1 position [1], which makes it a highly attractive target for therapeutic intervention. The conservation between the Mpros of SARS-CoV-2 and other betacoronaviruses has propelled efforts to develop broad-spectrum coronavirus protease inhibitors, since many of the previously identified SARS-CoV/MERS-CoV Mpro inhibitors were also active against SARS-CoV-2.
Figure 1

(a) Timeline of structure determination between January 2020 and March 2021. (b) SARS-CoV-2 genome organization. The nonstructural proteins are translated as two polyproteins that are processed by the two viral proteases, nsp3 (PLpro; cleavage site denoted by red star) and nsp5 (Mpro/3CLpro; cleavage site denoted by black arrowheads), resulting in 16 distinct proteins. The remainder of the genome encodes for viral structural (spike, envelope, matrix, and nucleocapsid) and accessory proteins encoded for by overlapping open reading frames. Nsp2-nsp16 assist in assembling or supporting the viral replication/transcription complex (RTC). (c) High resolution structures available for SARS-CoV-2 proteins. PDB IDs are shown in italic.

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Structure-based design of inhibitors against SARS-CoV-2 Mpro

There are currently over 250 high-resolution structures deposited for SARS-CoV-2 Mpro, including co-crystal structures with different protease inhibitors and fragment compounds [3**,4–6,7*,8–12]. These crystal structures have revealed that the substrate binding pocket is well defined, independent of whether a substrate was bound or absent (Figure 2c) [4–6,7*,8–12], supporting feasibility of a structure-guided inhibitor design approach. Investigations into Mpro blockers greatly benefited from the previously developed inhibitors of SARS-CoV Mpro that showed cross-inhibitory activity against SARS-CoV-2. Of these compounds, compound 11a and PF-07304814 are two of the most advanced Mpro inhibitors [7*,13*]. Both were originally developed using structure-guided approaches [7*,13*], and both are peptidomimetics. Co-crystal structures of each inhibitor bound to SARS-CoV-2 Mpro have been solved (Figure 2c) [7*,13*]. Compound 11a was originally designed based on the substrate-binding pocket of
SARS-CoV Mpro. A recent report showed compound 11a to be active in cell culture against SARS-CoV-2, revealing encouraging pharmacokinetics properties in three animal models (mice, SD rats, and beagle dogs) when administered intravenously [13]. However, some acute toxicity was observed in SD rats at high doses, and antiviral efficacy has not been confirmed in any SARS-CoV-2 animal model [13*]. Whereas these initial results are overall promising, compound 11a has yet to proceed to clinical trials. PF-00835231 was also developed using structure-based approaches to target SARS-CoV Mpro, and is capable of inhibiting SARS-CoV-2 in cell culture [7,14*]. Although likewise untested in animal models, initial pharmacokinetics results for PF-07304814, a phosphate prodrug of PF-00835231, were favorable and the compound is currently in a phase Ib clinical trial in hospitalized COVID-19 patients (NCT04535167). Although no SARS-CoV-2 Mpro inhibitor has succeeded in the clinic yet, data gleaned from current and previous SARS-CoV and MERS-CoV studies have provided a valuable template for how to successfully engage the Mpro active site using peptidomimetics [4–6,7*,8–12,13*,14*].

**Challenges of advancing structure-based Mpro inhibitors towards clinical use**

Besides the common challenges of traditional drug development (e.g. PK-ADME, efficacy, stability, formulation, route of administration), structure-based approaches face the added demand of time required to solve high-resolution structures. Extensive efforts must first be made to determine protein structures that lay the groundwork for structure-based design [3**]. A recent paper utilizing crystallographic fragment screening of SARS-CoV-2 Mpro underscores the time needed just to establish a foundation for fragment-based drug design [3**]. Although the authors generated a tremendous amount of structural data, the number of SARS-CoV-2 infections increased from ~40 0000 (Feb 9, 2020) to ~1 million global cases (April 1, 2020) in the amount of time required to release the first high-resolution structures. In addition, no fragment based Mpro inhibitor has since been developed, while the number of global cases has soared past 100 million. In short, the speed with which native structures of SARS-CoV-2 Mpro were solved is truly impressive and unprecedented in the history of infectious diseases, but despite these groundbreaking achievements no new inhibitor classes were identified in structure-guided drug development efforts that could contribute to halting the current COVID-19 pandemic.

**Nsp12 – the SARS-CoV-2 RNA-dependent RNA polymerase**

Viral RNA polymerases have been therapeutically targeted for the treatment of numerous viral infections, including those caused by HCV, HIV, and influenza virus [15–17]. In betacoronaviruses, viral RNA synthesis is coordinated by a multi-subunit replication/transcription complex (RTC) composed of a number of viral nonstructural proteins. Multiple high-resolution structures of SARS-CoV-2 RTC complexes have provided insight into the overall architecture of the RTC (Figure 3a) [18–22,23**,24,25*,26,27*,28*,29*,30–32,33*,34*]. The enzymatic functions of the complex are carried out by nsp12 (the viral RdRP), nsp13 (an RNA helicase/triphosphatase), nsp14 (a 3′ to 5′ exonuclease), nsp15 (an endoribonuclease), and nsp16 (an RNA cap methyltransferase) [18–22,23**,24,25*,26,27*,28*,29*,30–32,33*,34*]. Nsp12 constitutes the core of the RTC and represents one of the most promising druggable targets (Figure 3b–c) [30]. Underscoring this notion, remdesivir, the only direct-acting small molecule antiviral that has received FDA emergency approval against SARS-CoV-2 to date, targets the viral RdRP and is currently the standard of care (SOC) for treating severe COVID-19 [35]. Structures of the SARS-CoV-2 nsp12 complexes have been released, including apo and bound forms (Figure 3a,c) [22,24,25*,27*,28*,29*,30–32,33*,34*]. These data have provided great detailed insight into the mechanisms of RNA synthesis and potential means of inhibiting RdRP activity, but have yet failed to aid the structure-based identification of SARS-CoV-2 polymerase inhibitors. However, they have greatly advanced the molecular understanding of previously discovered RdRP inhibitors, in particular nucleoside/nucleotide analogs (NAs).

**Structural understanding of pharmacological Nsp12 inhibition**

Currently, the only promising antiviral therapeutics and drug candidates that target the SARS-CoV-2 RdRP are NAs, such as remdesivir, favipiravir, and molnupiravir (Clinical trials: NCT04336904, NCT04464408, NCT04529499, NCT04474457, NCT04434248, NCT04575584, NCT04575597, NCT04405739, NCT04405570). In the past year, several SARS-CoV-2 RdRP structures in complex with different NAs have been determined, including those of nsp12 in complex with an RNA primer-template incorporating remdesivir [25*,27*,28*,29*] or favipiravir [33*,34*]. These structures have revealed how favipiravir and remdesivir engage the RdRP and incorporate into the newly synthesized RNA. Although there is no precedent yet for any NA being developed using structure-guided approaches against any virus, these high-resolution structures may provide interesting starting points for future structure-guided development campaigns.

**Challenges of developing NAs as RdRP inhibitors against SARS-CoV-2**

To date, no allosteric SARS-CoV-2 RdRP inhibitors have been developed and advanced to clinical testing. For NA inhibitors, the SARS-CoV-2 exonuclease (nsp14) poses a potential problem, due to proofreading and excision of NAs such as ribavirin and 5-fluorouracil, from the viral RNA [36–40]. The ability of remdesivir to overcome SARS-CoV-2 exonuclease activity is likely due to its delayed chain termination mechanism of action, which
SARS-CoV-2 nsp12 RdRP. (a) Architecture of the nsp7-nsp8-nsp12-nsp13 RTC complex (PDB ID: 6xez). This complex includes nsp12 (green), nsp7 (orange), double stranded RNA (cartoon ribbon), two copies of nsp8 (pink and tan) and two copies of nsp13 (blue). (b) 2D-schematic of the SARS-CoV-2 nsp12 domain organization. (c) Structure of SARS-CoV-2 nsp12 bound to and RNA-primer template (PDB ID: 7l1f). Nsp12 can be broken down into two catalytic domains, a N-terminal Nidovirus-unique RdRP-associated nucleotidyltransferase (NiRAN; tan) domain and C-terminal RdRP domain connected by a central interface domain (purple). The RdRP domain forms a typical right-handed fold with fingers (green; residues 366-581 and 621-679), palm (teal; residues 582-620 and 680-815), and thumb (pink; residues 816-910) subdomains. Zinc and magnesium ions are shown as blue and green spheres, respectively.
blocks polymerase translocation three nucleotides after incorporation [27,28,41]. Since several nucleotides would need to be removed to excise the incorporated form of remdesivir (GS-441524), the drug may partially avoid removal by the SARS-CoV-2 exonuclease. In support of this hypothesis, it has been observed that the rate of incorporation of GS-441524 is greater than the rate of excision by the exonuclease [42]. Identification of nucleoside analogs that can avoid detection by the SARS-CoV-2 exonuclease might boost the success of NA therapies. Favipiravir [43,44] and molnupiravir [45] both utilize an alternate mechanism, induction of viral error catastrophe, to inhibit viral replication, albeit favipiravir is reportedly thought to act as a chain terminator also in some cases [46]. These two NAs are thought to avoid excision by the SARS-CoV-2 exonuclease, since both are rapidly incorporated into newly synthesized viral RNA and have been shown to induce lethal mutagenesis [43–45,47–51]. Interestingly, sequencing of SARS-CoV-2 infected hamsters treated with favipiravir did reveal mutations in the SARS-CoV-2 exonuclease, which could represent the development of resistant virus populations [52].

Two high-resolution cryo-EM structures of the SARS-CoV-2 RdRP in complex with favipiravir were recently solved [33∗,34∗]. In these structures, favipiravir forms non-canonical base-pair interactions, providing an explanation for how it can be incorporated as either adenosine or guanosine. Of note, favipiravir was bound in a non-productive binding mode, which could account for the low potency observed in vitro (EC50 = 118–207 μM) and in vivo (1000 mg/kg b.i.d. in Syrian hamsters) [33∗,34∗,43,53]. Results from clinical studies are ambiguous but tend to suggest that treatment with favipiravir may offer some benefit against COVID-19 [54–59]. However, study design and validity of the results of at least one of these trials are a subject of debate [60,61]. Molnupiravir has demonstrated oral efficacy against SARS-CoV-2 in multiple animal models [62–65], was found to be safe for human use in phase I clinical trial [66], and is currently in advanced phase II/III clinical trials. Unlike favipiravir, however, there are currently no high-resolution structures of the SARS-CoV-2 RdRP complexed with molnupiravir.

To date, there are also no high-resolution structures for the SARS-CoV-2 exonuclease alone or in complex with other components of the RTC. Therefore, a key piece of information is missing for the structure-guided development of NA therapies against SARS-CoV-2. Until it is understood how the SARS-CoV-2 exonuclease recognizes incorporated NAs in newly synthesized RNA, it will be difficult to use structure-guided approaches to proactively design next generation analogs that are resistant to detection and excision.

**The SARS-CoV-2 spike protein**

The SARS-CoV-2 spike (S) protein is a homo-trimeric envelope glycoprotein that is responsible for receptor binding and fusion of the viral envelope with host membranes [67]. The S protein is a type I fusion protein and exists in a metastable prefusion state, which can refold into an energetically far lower stable postfusion conformation [67]. A hallmark of type I fusion proteins is synthesis as an inactive precursor protein that must be proteolytically matured into two major subunits. In the case of SARS-CoV-2 maturation occurs predominantly by furin or host TMPRSS2 cleavage into S1/S2 and S2’, respectively (Figure 4a), to gain membrane fusion activity [67]. Of the proteolysis products, S1 is entirely extracellular and mediates receptor binding, whereas transmembrane S2 induces membrane merger [67]. The predominant cognate receptor of SARS-CoV-2 is human angiotensin-converting enzyme 2 (hACE2), which is recognized by the S protein receptor-binding domain (RBD) located in S1 [68,69,70∗,71]. Viral attachment leads to endocytotic uptake of the virion and ultimately activation of the fusogenic S2 subunit, which undergoes deep-seated structural changes that result in shedding of the S1 subunit and insertion of the fusion peptide into the host cell membrane, followed by S protein refolding into a hairpin-like structure and opening of a fusion pore [72–74].

**SARS-CoV-2 S is the primary target of protective humoral immunity**

The ability of the host to generate a robust antibody response against the SARS-CoV-2 S protein, in particular against S1, is critical for virus neutralization and effective immunoprotection [67,71,75–84]. Therapeutic antibodies targeting the SARS-CoV-2 S protein have been granted emergency use authorization by the FDA for the treatment of COVID-19 patients (Table 1). Structural studies mapping SARS-CoV-2 neutralizing epitopes identified two major sites for neutralizing antibodies (nAbs) derived from convalescent patient sera; the S1 N-terminal region [79,85,86] and the RBD [67,70∗,80,81,87] (Figure 4a-b). Of particular importance are antibodies targeting the RBD, based on their potential for broad-spectrum activity against other betacoronaviruses, including MERS-CoV and SARS-CoV [67,87–92]. A detailed structural understanding of the conserved epitopes or binding motifs recognized by these broad-spectrum antibodies could potentially be utilized to ultimately design a universal vaccine capable of providing protection against multiple coronaviruses.

**Importance of SARS-CoV-2 S in vaccine design**

Efforts to stabilize the pre-fusion state are critical for effective vaccine design, since important neutralizing motifs are present in the S1 subunit. Historically, stabilization of type-1 fusion proteins has been implemented for numerous viral species using a wide array of techniques, such as modifying cleavage sites, introducing disulfide bonds, and stabilizing flexible regions important for the conformational change between the pre-fusion and post-fusion states [93–100,101∗,102,103]. Structure-guided strategies have been applied to some of the current SARS-CoV-2 vaccines to stabilize S in a pre-fusion state.
and enhance the vaccine induced antibody response against the RBD and neutralizing epitopes in S1 [104,105]. Previous structure-based studies on the fusion proteins of HIV-1 and respiratory syncytial virus identified that proline substitutions in specific regions of the S2 subunit can hinder the conformational change to a post-fusion state [93–95].

Structural studies analyzing homologous regions of the S proteins of MERS-CoV and SARS-CoV identified two proline substitutions in S2 (S-2P; residues 986 and 987 in SARS-CoV-2 S) that likewise stabilize the S protein in a pre-fusion state [104] (Figure 4b). These mutations trap the S protein in an antigenically favorable pre-fusion state that induces an improved neutralizing antibody response compared to standard soluble S protein delivered, for instance, as a subunit vaccine. Both currently approved mRNA vaccines (the Moderna/NIAID and BioNTech/Pfizer vaccines) as well as the approved adenovirus-vectored Ad26.COV2.S (Janssen Pharmaceutical; FDA emergency use authorization on Feb. 27, 2021) utilize the S-2P strategy to stabilize the pre-fusion state of the SARS-CoV-2 S protein, resulting in neutralizing antibody titers that are equal to or greater than those of convalescent patients [106–109].

By comparison, the Oxford/AstraZeneca vaccine (ChAdOx1) uses a wild-type S protein without any stabilizing modifications. Of note, South Africa recently halted use of the Oxford/AstraZeneca vaccine due to less efficient protection against recently emerged mutant viral variants (https://www.nytimes.com/live/2021/02/07/world/covid-19-coronavirus). Currently, it is unclear whether a causal link exists between S protein design in the

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**Figure 4**

**Table 1**

| Monoclonal antibodies granted emergency use authorization (EUA) for the treatment of COVID-19 |
|-----------------------------------------------|
| Name                           | Target                  | EUA date  |
|-----------------------------------------------|
| Bamlanivimab + Etesevimab         | SARS-CoV-2 spike      | Feb-21    |
| REGEN-COV (Casirivimab + Imdevimab)  | SARS-CoV-2 spike      | Nov-20    |
AstraZeneca vaccine and this lower level of protection. However, antibodies isolated from recipients of the mRNA vaccines were only slightly less efficient in protecting against the E484K SARS-CoV-2 variant currently circulating in South Africa [110–112,113,114,115,116].

Since the initial design of SARS-CoV-2 vaccine candidates utilizing S-2P, several studies have identified and structurally characterized additional substitutions in S that led to higher levels of expression and greater thermostability compared to S-2P [117–120]. However, these new substitutions have not advanced to any candidates in clinical trials.

Limitations associated with SARS-CoV-2 structural data towards vaccine design

While previous structural studies on SARS-CoV and MERS-CoV have contributed greatly to the development of successful SARS-CoV-2 vaccines, structural data on SARS-CoV-2-S itself have provided limited novel information to enable vaccine design. However, high-resolution data has advanced the molecular understanding of the mechanisms of receptor binding, antibody mediated neutralization, and viral escape from neutralizing antibodies by recently identified SARS-CoV-2 variants. The previously identified S-2P modifications in SARS-CoV and MERS-CoV S structures have created a critical knowledge base for the rapid design of anti-SARS-CoV-2 vaccine candidates that were advanced in record time to clinical use.

Conclusions

High-resolution structural data have had an immense impact on our understanding of SARS-CoV-2 biology and can, with sufficient time, help advance antiviral and vaccine development [7*,13*,14*,104*]. It is important not to forget, however, that there is no direct short-cut path from structural knowledge to the rapid creation of a novel antiviral drug or vaccine. For example, structure-guided design originally targeting SARS-CoV and MERS-CoV has assisted in the development of SARS-CoV-2 Mpro inhibitors and conformationally stabilized SARS-CoV-2 S vaccine candidates. Similarly, the high resolution cryo-EM structures of nsp12 complexed with different NA inhibitors have illuminated the mechanism of action of different NAs, but originally other viral indications were targeted and development of these drugs dates back far before the COVID-19 pandemic [45,121,122]. An overarching challenge facing de novo structure-guided design of vaccines or drugs in the face of a mounting pandemic is the amount of time required to obtain high resolution structural data and translate this information to tangible clinical candidates. A major lesson learned from the COVID-19 pandemic is that a rapid first-line response is critical to limit virus spread. With current technology, this challenge can only be met when it is possible to build on a rich trove of pre-existing data generated for related viral pathogens. Even so, structure-guided drug design is unlikely to deliver a clinical candidate with the turn-around time required to impact the spread of a pandemic. Accordingly, no structure-guided antiviral specifically targeting SARS-CoV-2 has advanced to clinical use. However, available structural data have greatly shortened the timeline to vaccine development and approval, providing a tangible example of how proactively establishing a solid scientific foundation can prepare against an unexpected pandemic threat.

Conflict of interest statement

Nothing declared.

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