An Evolving Landscape of Small Molecules Targeting SARS-CoV-2: What Are We Awaiting Beyond 3CLpro Inhibitors?

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Abstract: It has been more than five years since the outbreak of SARS-CoV-2 pandemic. Today, approved drugs for treatment of coronavirus infection include Nirmatrelvir (Paxlovid), Molnupiravir, and Remdesivir. However, the continuous evolution of the virus underscores a need for the development of new therapeutic entities to complement with current front-line treatment. This Editorial focuses on recent breakthrough in the identification of novel NSP14 (Meyer *et al* Nature, 2024) and viral membrane protein inhibitors (Damme *et al*, Nature, 2025) of SARS-CoV-2. The inhibitors exploited previously unprecedented mechanisms for inhibiting their targets and viral replication, paving a potential path forward for uncovering new therapeutics against future pandemic. We opine that these groundbreaking discoveries warrant further discussion and insight from a wider community encompassing virologists, biochemists, and medicinal chemists. We also highlight the significance of the discovery and its potential impact for transforming the landscape of future therapeutic interventions against coronavirus and other RNA viruses.

Beginning in December 2019 in Wuhan, China, the COVID19 pandemic caused by SARS-CoV-2, rapidly spread globally and resulted in multi-million death cases. SARS-CoV-2 is a positive-sense single stranded RNA virus that utilizes its genome to replicate inside human cells, employing a combinatorial process involving viral and host machinery. Although Paxlovid/Ritonavir was approved for the treatment for SARS-CoV-2, concerns were raised due to the potential drug resistance through mutations within the viral 3CL protease [1]. A recent study showed that Paxlovid did not appear to benefit patients with long COVID from a 15-day regimen [2]. A second generation 3CLpro inhibitor Ibuzatrelvir is currently under phase 3 trial as a single oral agent. Nucleoside based inhibitors such as Molnupiravir, on the other hand, is mainly approved for severe cases [3]. These highlight the need for alternative therapies to combat the continuously evolving virus, ideally via a combination/cocktail therapy at early phase of infection. One potential strategy is to pair Paxlovid with inhibitors targeting other viral proteins, such as structural proteins and the replisome **(Figure 1**) including NSP12 (RNA-dependent RNA Polymerase), NSP13 (helicase), and NSP14 (RNA methyltransferase). While Remdesivir [4] is approved for treatment of patients hospitalized for COVID19, its clinical benefit remained controversial [5]. Despite significant progress in identification of NSP13 inhibitors [6], no compound has advanced to clinical stage yet. Another critical target is NSP14, a key protein involved in RNA capping for the viral genome.[7] This process adds a 7-methylguanosine (m7G) cap to the 5' end of the viral RNA, a protective modification that prevents RNA from degradation[8]. Mechanistically, NSP14 binds to the viral RNA and, with the presence of its cofactor, S-adenosylmethionine (SAM), transfers the methyl group from SAM to the guanine nucleotide at the 5' end of the RNA, leading to the formation of the methylated cap (**Figure 2**). In addition, NSP14 also ensures RNA stability and helps evade the host's immune defences by preventing the RNA from [9]. As such, the multiple functions of NSP14 [10] make it an attractive target for antiviral therapies. Unfortunately, NSP14 is a challenging target for drug discovery due to its low druggability, primarily attributed to the physicochemical properties of both the substrate and co-factor binding sites, being relatively flat and extremely hydrophilic, making it difficult to identify drug-like small molecule inhibitors for advancing to preclinical setting.

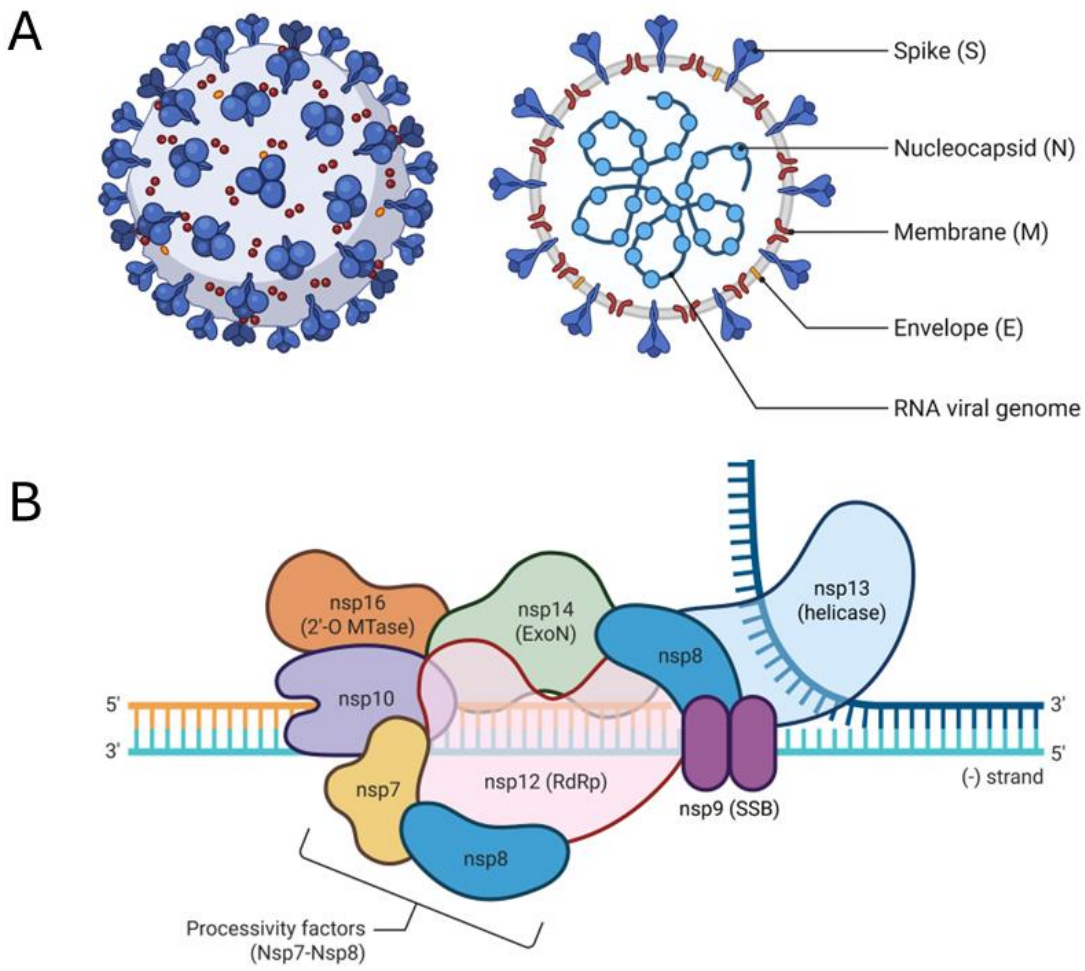


Figure 1. Cartoon of the structure of SARS-CoV-2 and possible drug targets within the viral genome. A) Human coronavirus structure and key structural proteins on the viral surface. B) Cartoon illustration of a model of putative coronavirus replisome consisting of a multi-protein molecular machine responsible for the viral RNA replication and transcription. The figure was created using Biorenderhttps://BioRender.com

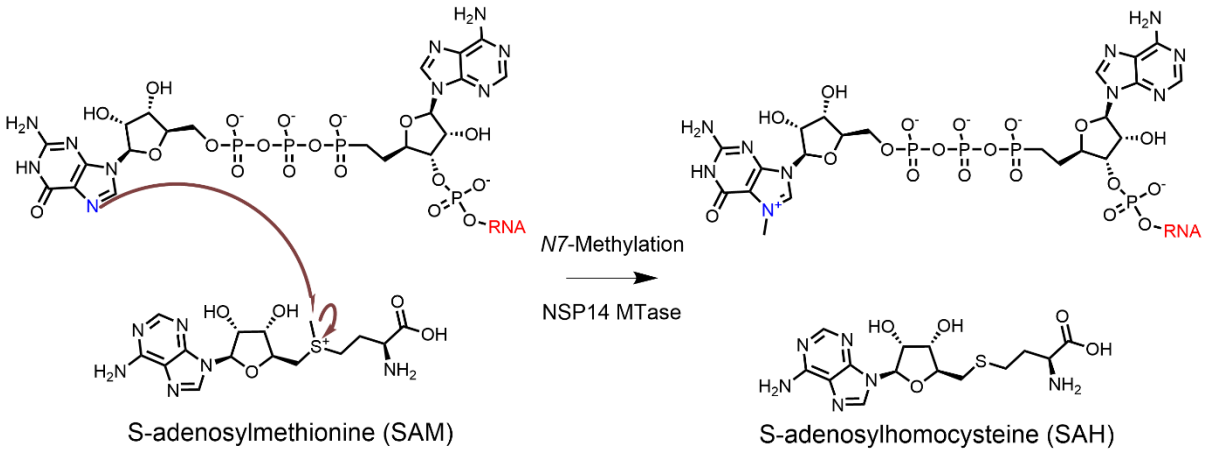


Figure 2. Mechanism of NSP14 in RNA capping. The N7 atom is highlighted in blue.

Past drug discovery efforts to develop NSP14 inhibitors led to identification of both SAM-analogues and non-cofactor based small molecules [11]. Only until recently, a report in ***Nature*** revealed an unprecedented NSP14 inhibitory mechanism, offering a new direction to overcome the challenges associated with inhibiting this enzyme [12]. From the study, Meyer *et al* employed a high-throughput screening and identified a hit molecule RU-0415529, which inhibited SARS-CoV-2 NSP14 with an IC_{50} of 356 nM. Further optimization resulted in TDI-015051 which forms a tight complex with NSP14 (Kd = 61 pM) in the presence of SAH. A cell-based assay revealed a half-maximal effective concentration (EC_{50}) of 11 nM for TDI-015051. Biochemical and biophysical characterization showed that the inhibitor adopted an unusual inhibitory mechanism by establishing a ternary S-adenosylhomocysteine (SAH)-bound NSP14 complex. X-ray crystallography revealed that TDI-015051 binds in close vicinity to SAH/SAM by accommodating the cap-binding pocket (**Figure 3**). TDI-015051 formed notable interactions with NSP14 and SAH, including a hydrogen bond with the hydroxyl group of Thr428 sidechain, a hydrogen bond with the carboxylic acid from SAH, and a cation-π interaction with Arg310. By "glue-ing" SAH, the inhibitor essentially locked NSP14 in a conformation that prevents the dissociation of reaction products, maintaining the integrity of a specific molecular architecture that would otherwise lead to product release, therefore impairing the RNA capping function. Oral administration of TDI-015051(150 mg/kg and 500 mg/kg) resulted in robust reduction of the viral lung burden in a K18-hACE2 transgenic mouse model. Despite the liability from inhibiting CYP3A4 (IC50 = 0.02 μM), which might affect its clearance *in vivo* and potential cause for drug-drug interactions, the proof-of-concept study shed light on a novel inhibitory mechanism of NSP14, paving a new avenue toward drugging the notoriously intractable target.

Beyond targeting replisome-associated proteins, structural proteins of SARS-CoV-2 presents another viable avenue for small molecule drug discovery, as they participate in viral assembly, replication, and immune evasion. These include the spike (S), envelope (E), membrane (M), and nucleocapsid (N) protein. A recent publication reported a unique mechanism whereby a small molecule inhibited the M protein of SARS-CoV-2 [13]. The viral membrane protein of SARS-CoV-2 plays a key role in viral assembly and release. Canonically, M protein forms a homodimer and samples a conformational equilibrium between an elongated form and a short form. Both states of the M protein are essential to its role involved in viral assembly. Researchers from Janssen Pharmaceutica employed a high-throughput screen and identified JNJ-9676 as an M protein inhibitor, which unexpectedly induced significant conformational changes of the protein, resulting in a structural state that is incompatible with viral assembly (**Figure 4**). The compound adopts a bent into an "L" shape and binds to an induced pocket wedged between TM2 and TM3 of one of the protomers, engaging in hydrophobic and H-bond interactions with surrounding residues.

The seminal success of above work is tied to several key factors: First, high-throughput screening (HTS) continues to play an important role in identifying unique inhibitors that might otherwise be missed, supporting HTS represents an attractive tool in early drug discovery pipeline. Second, the mechanistic characterization of the hit via a combination of biochemical, biophysical and structural methods is critical to the understanding of the molecular recognition for validating the novel mechanism that may guide future hit-to-lead medicinal chemistry effort toward the optimization of the novel hit scaffolds. While drug discovery targeting NSP14 of SARS-CoV-2 has entered a new milestone, it may hold promise that this approach could potentially be adapted to other viral proteins with similar mechanisms. One example is the NSP16 from coronavirus, which is also involved in RNA capping, specifically the 2'-*O*-methylation of the RNA cap. Moreover, the NS5 protein in flaviviruses (e.g., Zika, Dengue, West Nile) is responsible for both RNA capping and RNA-dependent RNA polymerase activities. Finally, the neuraminidase from influenza virus cleaves glycosidic bonds to remove sialic acid from the surface of host cells, facilitating virion release. Sharing a common ground with NSP14, these proteins should receive consideration for exploring the current approach to unlock the untapped territory for drugging them. The available co-crystal structure of substrate/cofactor bound ternary complexes provides a template for future structure-based drug design, enabling computational screening to be applied to develop novel inhibitors from ultra-large chemical space. On the other hand, the structural pocket bound by JNJ-9676 provides a

template for future drug discovery effort toward the sought of a membrane protein inhibitor of SARS-CoV-2. The success in targeting a viral structural protein also presents a promising strategy that could be extended to other viruses. With the evolving understanding of novel mechanisms that may be leveraged to inhibit functional protein targets from SARS-CoV-2, we will await a transformative shift in the landscape of antiviral therapy. It is conceivable that small molecules targeting previously underexplored mechanism will fuel the next wave of clinical candidates for the treatment of coronavirus and other RNA viral infections.

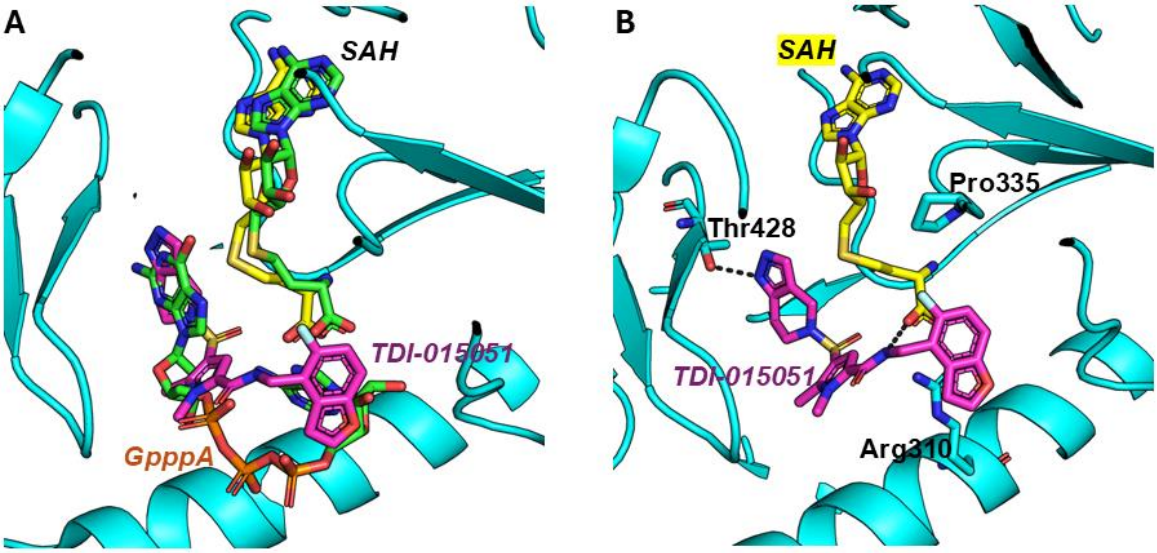


Figure 3. Structures of NSP14-bound to SAH, GpppA, and TDI-015051. A) Superimposition of crystal structures from SARS NSP14-GpppA-SAH complex (PDB: 5C8S) and SARS-CoV-2-NSP14-TDI-015051-SAH complex (PDB: 8R7B). TDI-015051 (purple) partially overlaps with GpppA within the substrate binding pocket. B) Molecular mechanism of TDI-015051 in inhibiting NSP14 via the stabilization of the byproduct SAH. H-bond is depicted in black dotted line.

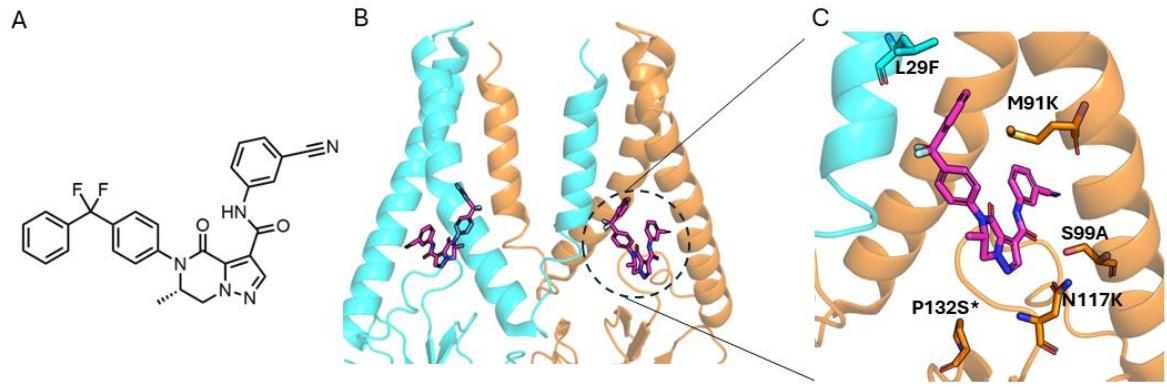


Figure 4. A) Molecular structure of JNJ-9676. B) The cryo-EM structure of M protein structure (homodimer) bound to JNJ-9676. C) Zoom in view of binding mode of JNJ-9676 in M protein of SARS-CoV-2, with binding site residue mutations identified from *in vitro* resistance selection assay labelled. Each monomer of M protein is shown in cyan and orange, respectively.

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