Special Report

Future Drug Discovery

Overcoming nonstructural protein 15-nidoviral uridylate-specific endoribonuclease (nsp15/NendoU) activity of SARS-CoV-2

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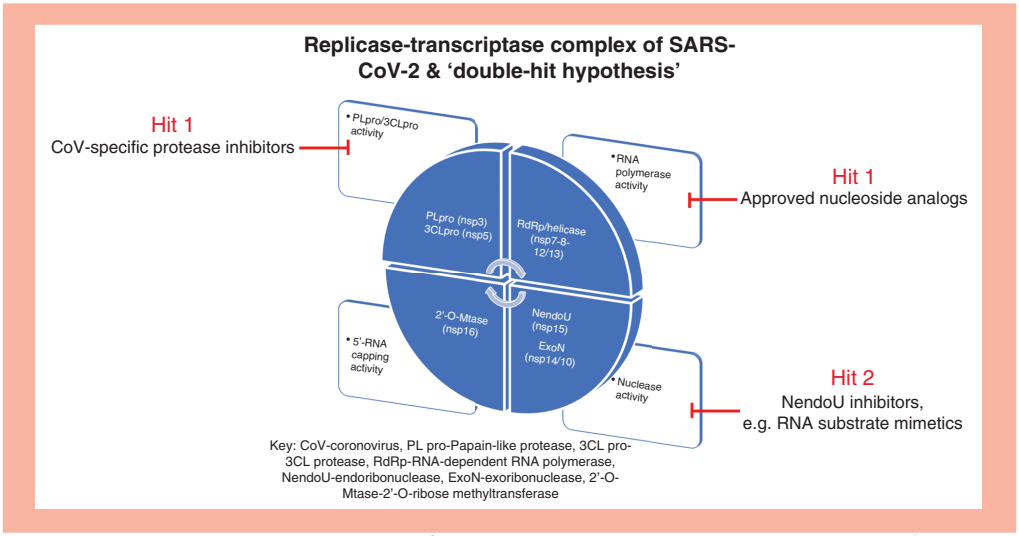
COVID-19 has become the gravest global public health crisis since the Spanish Flu of 1918. Combination antiviral therapy with repurposed broad-spectrum antiviral agents holds a highly promising immediate treatment strategy, especially given uncertainties of vaccine efficacy and developmental timeline. Here, we describe a novel hypothetical approach: combining available broad-spectrum antiviral agents such as nucleoside analogs with potential inhibitors of NendoU, for example nsp15 RNA substrate mimetics. While only hypothesis-generating, this approach may constitute a 'double-hit' whereby two CoV-unique protein elements of the replicase–transcriptase complex are inhibited simultaneously; this may be an Achilles' heel and precipitate lethal mutagenesis in a coronavirus. It remains to be seen whether structurally optimized RNA substrate mimetics in combination with clinically approved and repurposed backbone antivirals can synergistically inhibit this endonuclease *in vitro*, thus fulfilling the 'double-hit hypothesis'.

Graphical abstract:



Replicase–transcriptase complex of SARS-CoV-2 & 'double-hit hypothesis' (Key: CoV-coronavirus, PLpro-Papain-like protease, 3CLpro-3CL protease, RdRp-RNA-dependent RNA polymerase, NendoU-endoribonuclease, ExoN exoribonuclease, 2′-O-Mtase-2′-O-ribose methyltransferase)

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As of early May 2020, there is no specific anti-COVID-19 therapy available (clinical management of severe acute respiratory infection when COVID-19 disease is suspected; WHO interim guidance version 1.2). We, among others, have posited that a combination strategy with 'repurposed drugs' could offer the best chance of combating the current COVID-19 pandemic gripping the world [1]. In other words, 'multiple drugs versus one virus' where different nodes of the virus–host interactome are simultaneously targeted with antivirals to achieve a synergistic effect [2]. Indeed, the case for combination antiviral therapy is becoming increasingly clear, as demonstrated by the modest clinical efficacy hitherto reported of monotherapy, including for the leading RdRp inhibitor remdesivir [3].

Such a strategy is not without historical precedence in modern medicine, even before the era of 'personalized medicine', the latter best exemplified by precision oncology. For example, HIV causing AIDS rapidly emerged into the global scene in a very similar pandemic-like fashion during the late 1980s and 1990s. The ensuing decade saw the utilization of this combination strategy with highly active antiretroviral therapy against the new HIV virus. The success of this is evident and AIDS is now considered a manageable, chronic condition if addressed early on in its natural history. As the scientific community scrambles to find a therapeutic solution to face the latest global viral threat in the form of SARS-CoV-2, it is prudent to remember this history's lesson and attempt to replicate it. As such, what could be novel approaches to realize combination therapy in the context of COVID-19?

Rationale for the double-hit hypothesis

To appreciate the multiple drugs versus one virus approach, we must first understand the dynamics of the 'host–virus protein–protein interactome'. Using integrative meta-analysis, Bösl *et al.* have demonstrated a glimpse into viral evasion mechanisms that target central nodes of the host–virus interactome [2]. Here, a seemingly 'hub-and-spoke model' of the host–virus protein–protein interactome emerges in which viruses across families and strains ubiquitously target core host proteins involved in multifunctional cellular pathways for maximal effect. This is of particular importance to smaller RNA viruses (as compared with DNA viruses) with more reliance on host cellular machinery to partly compensate for their higher mutation rate per strand copying [4]. RNA viruses such as SARS-CoV-2 rely on RNA-dependent RNA polymerases (RdRps) for faithful transmission of their genomes from host to host [5]. However, RdRps are known for their low fidelity of replication, resulting in an elevated rate of spontaneous mutations in RNA viruses [6]. Consequently, nucleoside analogs have become a backbone of modern antiviral therapy, exploiting base misincorporation into RNA viral genomes for their therapeutic effect.

Ribavirin is a nucleoside analog broad-spectrum antiviral agent (BSAA) with activity against a number of RNA and DNA viruses [7]. It is one of the longest standing antiviral agents included in the WHO's Model List of Essential Medicines (21st list, 2019). These rather promiscuous antiviral properties of ribavirin have been explained by at least five mechanisms; indirect mechanisms, including inosine monophosphate dehydrogenase inhibition affecting the cytoplasmic nucleotide pool and immunomodulatory properties, and direct mechanisms, including RNA capping interference, canonical RdRp inhibition and lethal RNA viral mutagenesis causing 'error catastrophe' [8,9]. However, ribavirin has not demonstrated a significant clinical benefit during previous outbreaks of *coronaviridae*, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), upon meta-analysis [10–12]. This is corroborated by ribavirin's IC_{50} in the high micromolar range against human CoVs *in vitro*, which currently limits its use in this setting due to toxicity at pharmacologically potent doses [13,14]. Notably, this is in contrast to ribavirin's efficacy particularly in combination therapy against a wide variety of viral infections including hepatitis C virus, respiratory syncytial virus and Lassa fever [15–17].

Why did a model BSAA with a repertoire of antiviral mechanisms against a number of RNA viruses fail in the face of previous human coronavirus respiratory syndromes? Briefly, work of Ferron *et al.* using SARS-CoV-1 offers an explanation how a coronavirus can overcome lethal mutagenesis with an endowed exonuclease (ExoN) activity by RNA nucleotide mismatch correction [18]. Albeit *in vitro* evidence, such observations may explain the proven efficacy of a broad-spectrum nucleoside analog against hepatitis C virus without any exonuclease activity and lack of efficacy against SARS or MERS in possession of exonucleases. However, it is perhaps important that we reassess to explore any other overlooked strategies that could potentiate the efficacy of a BSAA in general.

Extrapolating from SARS-CoV-1 & MERS-CoV

Rapid sequencing efforts have established that SARS-CoV-2 is approximately 80% identical to SARS-CoV-1 at the whole genome level [19,20]. This similarity apparently extends to the pathophysiological level of viral replication cycle to some degree, where SARS-CoV-2 shares the same angiotensin-converting enzyme 2 cellular receptor for

entry and primes its spike (S) protein with the transmembrane protease serine 2 [19,21–23]. The viral genome can be broadly segmented into regions consisting of nonstructural, structural and accessory genes. Importantly, homology modeling of four key viral proteins (papain-like protease, 3CL protease, helicase and RdRps) suggest that active sites of these viral enzymes to be highly conserved across the three known human coronaviruses (CoVs) with pandemic potential [24,25]. As such, it is a reasonable inference to extrapolate the pharmacology of antiviral agents previously tested against SARS and MERS to rationalize therapeutic options for COVID-19.

Out of the three viral gene regions, those encoding the 16 nonstructural proteins (nsps) perhaps reign supreme in the hierarchy of critical importance to the viral life cycle, as they function in the interphase between cellular entry and exit facilitating ongoing transmission of the virus. Many of these nsps assemble into a supercomplex known as the replicase–transcriptase complex (RTC) with multienzymatic properties that is critical for replication and transcription of subgenomic RNAs [26]. Even more unique to CoVs are the nsp15-NendoU and nsp14-ExoN ribonucleases (the former in particular), which can be considered as genetic markers of the *Nidovirales* order, distinguishing it from all other RNA viruses [18,27]. The work of Ivanov *et al.* in 2004 following the SARS outbreak perhaps best exemplified the indispensability of nsp15 to a human CoV. A single-nucleotide mutation of *nsp15* abolished its endonucleolytic activity and viral RNA synthesis. This observation has been corroborated further *in vivo* where CoVs expressing mutant nsp15 forms resulted in early robust induction of interferon, apoptosis of macrophages and stimulated a protective immune response leading to significantly attenuated disease in murine models [28]. Therefore, this endonuclease can be considered an Achilles' heel-type weaknesses and specific antiviral target [29]. However, other studies have indicated that the endoribonuclease activity of CoV nsp15 can be dispensable for the viral replication cycle, albeit required for optimal infection [30,31]. In fact, the key contribution of this evolutionarily conserved endoribonuclease may be the efficient evasion of the innate immune response at the very site of RNA synthesis during the early phase of infection [28,32]. By functioning as an interferon antagonist and evader of double-stranded RNA sensors in macrophages, nsp15 likely represents a primary strategy employed by a CoV to evade the intrinsic antiviral responses and gain a foothold in the host [33].

The S protein on the contrary, while crucial for initial viral entry into target cells, has been observed in some CoVs to directly transit to the cell surface without being assembled into virions prior to exocytosis [34]. There it mediates fusion between infected and uninfected cells leading to the formation of giant, multinucleated cells facilitating almost a 'paracrine mode of viral spread' in a target tissue. If this is a mechanism faithfully employed by SARS-CoV-2, there could be significant ramification for vaccine development, which represents a major focus of current global efforts. The S protein has intuitively received most attention as the best target for development of monoclonal antibodies. However, the aforementioned mechanism of viral spread could become implicated in circumventing detection and neutralization by antibodies.

Case for inhibiting nsp15/NendoU

The endoribonuclease activity of nsp15/NendoU involves uridylate-specific cleavage of RNAs [29,35–37]. Function of the CoV endonuclease in relation to its viral life cycle has remained somewhat elusive. Evidence exists in animal models for an immunomodulating property during early viral infection in addition to its canonical endoribonuclease activity. Nsp 15 (among other nsps) seems to play a dominant role in suppressing the type I IFN (IFN-α/β)-associated innate immune response by infecting macrophages, thus avoiding detection of viral mRNA by double-stranded RNA sensors [28,32,38]. Evidence from SARS animal models indicates that it is a delayed yet exuberant IFN-I signaling with an accumulation of neutrophils, monocytes and macrophages in lungs that is the major precipitant of lung immunopathology [39]. It is suggested that this temporally dysregulated and late-enhanced innate immune response with an influx of myeloid cells preceding a 'cytokine storm of proinflammatory mediators' drives acute respiratory distress syndrome, which is the dominant cause of COVID-19 mortality [40]. Site-directed mutagenesis of the homologous NendoU domain in equine arterivirus (type of nidovirus) has demonstrated up to five log reduction in viral replication and subgenomic RNA accumulation [41]. Being an endonuclease and a potential 'suicide enzyme' for an RNA virus, NendoU activity thus is evidently under tight control during replication [42]. *In toto*, nsp15 can be considered a highly specific and unique target for anti-CoV therapeutics.

RNA mimetics as decoy inhibitory ligands against nsp15/NendoU

A high-resolution crystal structure by x-ray diffraction for the nsp15/NendoU of SARS-CoV-2 has now been preliminarily reported leading to some crucial observations [43]. In their Protein Data Bank entry of SARS-CoV-2 nsp15/NendoU (PDB ID: 6VWW), Kim *et al.* report 88% sequence identity and 95% similarity with its closest

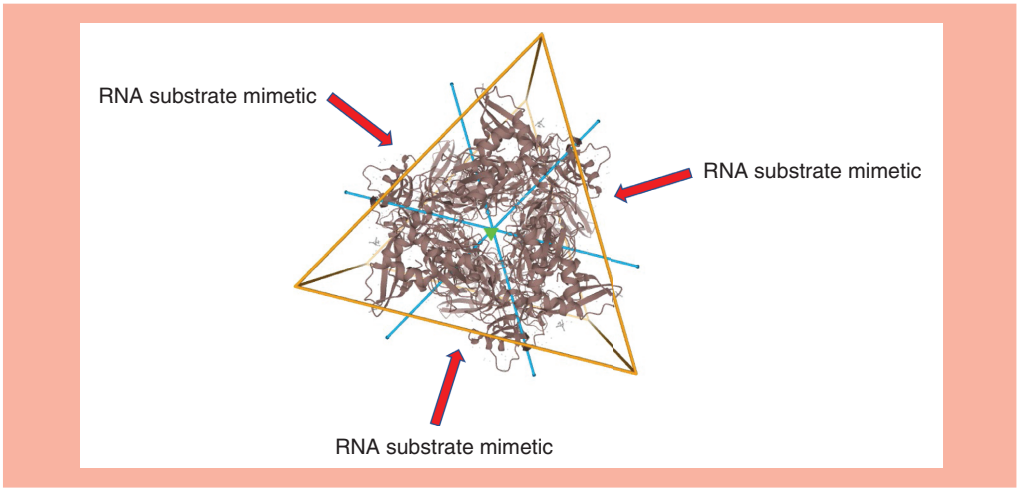

Figure 1. Inhibition of nsp15-NendoU from severe acute respiratory syndrome coronavirus-2 with RNA substrate mimetics. PDB ID: 6VWW Global Symmetry top view: arrows indicate the proposed RNA substrate mimetics as ligands and competitive inhibitors of the nsp15 monomers.
Crystal structure adapted from Kim *et al.* [43].

known homolog in SARS-CoV-1. MERS-CoV nsp15/NendoU, on the contrary, is somewhat more distant with only 50% sequence identity and approximately 65% similarity. More importantly, six key residues forming the catalytic site (His235, His250, Lys290, Thr341, Tyr343 and Ser294) are universally conserved among SARS-CoV-2, SARS-CoV-1 and MERS-CoV, both sequentially and conformationally. This suggests that all three homologs should have extremely similar if not identical substrate affinity. The functionally active form of nsp15 is a double-ring hexamer made of a dimer of trimers, which is consistent with structures of SARS-CoV-1 and MERS-CoV nsp15s [43]. Up to six preferentially single-stranded RNAs can engage the hexamer from top and bottom for cleavage (Figure 1). Therefore, inhibitors of SARS-CoV-1 nsp15 can be expected to inhibit its homolog in SARS-CoV-2. It must be noted that the work of Ivanov *et al.* [29], cited here for primarily supporting nsp15 as a unique nidoviral genetic marker, employed a set of mutant derivatives of HCoV-229E NendoU with Ala substitutions of conserved residues across XendoU/NendoU family. HCoV-229E is a related human CoV which is known to cause common colds and falls in the Alphacoronavirus genus; this contrasts with the Betacoronavirus genus of SARS-CoV-2. As the new nsp15/NendoU crystallographic data from Kim *et al.* demonstrates, high sequence identity or similarity cannot be expected between HCoV-229E and SARS-CoV-2 given the evolutionary distance [43]. Therefore, direct comparison of the active site residues from these studies is difficult and thus necessitates site-directed mutagenesis studies with SARS-CoV-2.

This brings us back to previous work by Bhardwaj *et al.* in 2006 [36]. Using SARS-CoV *in vitro* models, it was demonstrated that nsp15 specifically cleaves 3′ of uridylate in 16-nt, favorably modified RNA sequences. In a series of elegantly designed experiments, derivatives of the 16-nt prototype RNA substrate (named R16.4) that cannot be efficiently cleaved by nsp15 could still act as competitive inhibitors of this enzyme. These derivatives (e.g., PT13, mR16.4) obtained by minor modifications to the prototype R16.4 sequence (5′GAAGCGAAACCCUAAG3′) retain the ability to interact with nsp15, thus acting as competitive inhibitors of NendoU activity. Single-stranded RNA oligonucleotides with multiple, unpaired uridylate bases had preferentially higher affinity binding to nsp15.

This raises the possibility of a novel approach where RNA substrate mimetics of nsp15 can be deployed as endonuclease inhibitors. An important question is whether combining such endonuclease inhibitors with nucleoside analogs (as backbone antiviral agents) would prove to have an additive or synergistic effect against CoVs. A further possibility is a 'triple-therapy regimen' combining two approved nucleoside analogs, for example ribavirin and sofosbuvir, a combination shown to have tight RdRp inhibition in a recent molecular docking study [44].

Future perspective

The leading edge of current global efforts to curb the COVID-19 pandemic is understandably the development of a vaccine. The WHO reported in April 2020, that over 70 candidate vaccines are at different stages of the translational

process at an unprecedented speed. Indeed, a proof of principle has been demonstrated in a rhesus macaque model where recovery from infection with SARS-CoV-2 conferred immunity to rechallenge with the same strain [45,46]. However, recent phylogenetic network analyses on reported SARS-CoV-2 genomic samples from around the world have found at least three central variants (A, B, C) indicating in human clonal evolution with possibly different lethality [45,47,48]. First, most present vaccine candidates have a specific target epitope of SARS-CoV-2, and such rapid antigenic drift of the virus could seriously jeopardize effective vaccination. Second, even an effective preventive vaccine could only perhaps reduce the global infection rate to a basal level, meaning likely cyclical re-emergence of outbreaks similar to seasonal influenza in the near future. This poses a provocative question: is a situation in which vaccine composition has to be constantly changed to suit the dominant virulent strains practically feasible or desirable? All in all, this further highlights the critical role of designing novel therapeutic strategies with antivirals.

Here, we have attempted to elaborate on the previous proposal for using a BSAA combination therapy for COVID-19. Our focus was a concept for further investigation, in other words utilizing already licensed BSAAs with some activity against SARS-CoV-2 concurrently with experimental RNA substrate mimetics as nsp15/NendoU inhibitors. To our knowledge, only *in vitro* evidence exists for such nuclease inhibitors to-date [36]. More lead optimization work on such RNA substrate mimetics to further establish proof of principle is now warranted at a minimum. These can include parallel cellular assays to characterize their stability within microsomes or nanoparticles and off-target properties. As such, the 'double-hit effect' is hypothesis-generating at present and additional supporting data would be necessary prior to advancement of this strategy into *in vivo* animal model studies and subsequent first-in-human clinical trials. Conversely, the CoV nsp14 would be an attractive target to combine with a BSAA; inhibition of the ExoN catalytic site can be expected to knock out this viral RNA proofreader, thus restoring the potency particularly of nucleoside analogs that are readily available antiviral agents [1,18,42,47]. Unlike for NendoU, we are not yet aware of any reported inhibitor class specifically of the CoV exonuclease activity, which limits advancing this combination strategy for now.

We can only speculate on the challenges of RNA substrate mimetics vis-à-vis their translational potential. While the PK/PD profiles of long-standing nucleoside analogs are well established, the *in vivo* dynamics of potential RNA substrate mimetics are essentially unknown. Additionally, the optimal method or vehicle for their delivery remains to be determined. One possibility is the emerging RNA-based delivery system which can offer significant advantages in terms of simpler design and flexibility of manipulation in comparison to the more traditional viral subunit/vector-driven methods [49]. It is also noteworthy that mRNA is under investigation as a robust platform for at least one of the current leading vaccine candidates (mRNA-1273 by Moderna, Inc., MA, USA) which has rapidly been translated into Phase I clinical trial (ClinicalTrials.gov Identifier: NCT04283461). This involves a novel lipid nanoparticle-encapsulated mRNA formulation that encodes for the full-length S protein of SARS-CoV-2 which can be readily administered intramuscularly. In principle, given the relative ease of manufacturing RNA oligonucleotides compared with large recombinant viral proteins, we can anticipate quick adaptability of a similar delivery system to RNA substrate mimetics as cargo. Another important consideration is the temporality of administration of such antiviral therapy. As has been empirically noted, an initially dampened host innate immune response preceding a late exuberant cytokine storm are the hallmarks of severe COVID-19 immunopathology and associated mortality [50]. Given the current understanding of CoV-nsp15/NendoU as a major immunomodulator, counteracting viral evasion of host innate immunity in the preliminary stages of disease seems logical. Additionally, formation of double membrane vesicles and RTC activity dominate early during the coronavirus replication cycle before viral protein synthesis escalates to sufficient levels causing viremia and systemic infection. Therefore, the ideal time for inhibition of SARS-CoV-2 endonuclease activity within a combination strategy is perhaps quite early on in COVID-19.

Further, even if favorable PK/PD data are obtained through preclinical testing, the toxicity profiles of such therapeutics solo and in combination for drug–drug interactions would need to be rigorously evaluated in model *in vivo* systems. The 'double-hit hypothesis' may also dictate that SARS-CoV-2 proteases (i.e., PLpro and 3CLpro) could serve as the add-on third viral enzyme target on top of a backbone of RdRp inhibition with a nucleoside analog. On the contrary, 2′-O-Mtase activity, while possibly facilitating viral evasion of the host immune response through epigenetic RNA modification, may not be considered crucial for driving the replication cycle relative to other nsps of the RTC. Given no reported inhibitors of 2′-O-Mtase activity in SARS-CoV-2, we posit that this cannot be expected to rise to the significance of either hit 1 or 2 (see graphical abstract). Proteases also are not unique to the *Nidovirales* order *per se*; whether they can be of specific 'synthetic lethality' to *coronaviridae* when inhibited in combination remains to be seen.

Recent clinical trial data somewhat reinforces this doubt whether protease inhibition can be of significant benefit in the context of COVID-19. Lopinavir-ritonavir (protease inhibitor combination approved for HIV) did not produce any clinical benefit over standard of care in hospitalized adult patients with severe COVID-19 [51]. Upon reflection, this may not be surprising as lopinavir is designed to be an inhibitor of the retroviral protease which is different from the cysteine protease class of CoVs. While drug repurposing is a time-critical method, this experience should remind us of the fundamental principle of target specificity in preclinical development to enhance clinical success. Encouragingly, recent timely work has determined the crystal structure of SARS-CoV-2 main protease (Mpro) also known as 3CL protease (3CLpro). This in turn allowed the identification of potentially specific protease inhibitors with translational potential through a program of combined structure-based drug design, virtual and high-throughput drug screening [52,53]. It must also be acknowledged that there is no historical precedent for RNA substrate mimetics in translational medicine, which renders them in uncharted waters. Alternatively, small molecule inhibitors of RNaseA have been proposed as a strategy to inhibit coronavirus nsp15 as it bears crystal structural similarity to RnaseA [33]. However, as the initial suboptimal efficacy of repurposed HIV protease inhibitors for COVID-19 has demonstrated to some degree, target specificity and substrate affinity must remain paramount even in this brave new world of a pandemic. While RNaseA inhibitors can shut down the endonuclease activity, off target effects in the host would be a concern that needs *in vivo* evaluation. Therefore, the current *in vitro* evidence, though limited on RNA substrate mimetics for their potential as specific CoV endonuclease inhibitors, can still serve as the basis for further confirmatory studies to validate the concept.

In conclusion, we hope this insight adds a new layer to a fast expanding armamentarium of therapeutic strategies against COVID-19. Nsp15/NendoU RNA substrate mimetics described in previous studies deserve to be revisited for preclinical optimization and testing, perhaps in combination with nucleoside analogs [36].

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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