

Report of the National Institutes of Health SARS-CoV-2 Antiviral Therapeutics Summit

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The NIH Virtual SARS-CoV-2 Antiviral Summit, held on 6 November 2020, was organized to provide an overview on the status and challenges in developing antiviral therapeutics for coronavirus disease 2019 (COVID-19), including combinations of antivirals. Scientific experts from the public and private sectors convened virtually during a live videocast to discuss severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) targets for drug discovery as well as the preclinical tools needed to develop and evaluate effective small-molecule antivirals. The goals of the Summit were to review the current state of the science, identify unmet research needs, share insights and lessons learned from treating other infectious diseases, identify opportunities for public-private partnerships, and assist the research community in designing and developing antiviral therapeutics. This report includes an overview of therapeutic approaches, individual panel summaries, and a summary of the discussions and perspectives on the challenges ahead for antiviral development.

Keywords. SARS-CoV-2; antiviral therapeutics; emerging modalities; preclinical; proteases; viral replication machinery.

INTRODUCTION TO THE NIH VIRTUAL SARS-COV-2 ANTIVIRAL SUMMIT

The NIH Virtual SARS-CoV-2 Antiviral Summit was held on 6 November 2020. The Virtual Summit was organized to provide an overview on the status and challenges in developing antiviral therapeutics for coronavirus disease 2019 (COVID-19), including combinations of antivirals, and it was streamed live to allow broad public access while maintaining social distancing. Scientific experts from the public and private sectors convened to discuss severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) targets for drug discovery, and the preclinical

tools needed to develop and evaluate effective small-molecule antivirals. The goals of the Summit were to review the current state of the science, identify unmet research needs, share insights and lessons learned from treating other infectious diseases, identify opportunities for public-private partnerships, and assist the research community in designing and developing antiviral therapeutics.

The Summit was jointly organized by National Institute of Allergy and Infectious Diseases (NIAID), National Center for Advancing Translational Sciences (NCATS), and the National Institutes of Health (NIH) Office of the Director, and hosted by Dr Francis Collins (NIH), Dr Anthony Fauci (NIAID), and Dr Christopher Austin (NCATS). The meeting moderators and panelists were from academia, industry (pharma/biotech), NIH Institutes and Centers, and Federal agencies working in the COVID-19 therapeutics space.

The Summit itself comprised introductory remarks, an overview of SARS-CoV-2 replication and therapeutic targets, and 5

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scientific panels that followed. In each panel, the session moderator provided an overview of the topic (slides are included in the [Supplementary Material](#)) and facilitated discussion with panelists. An update on vaccines and neutralizing antibodies was provided, and a final summary session was held to identify key points from the Summit. The meeting agenda is shown ([Box 1](#)), and the principal session topics were:

- Viral replication machinery
- Proteases (viral and host)
- Emerging targets, emerging modalities
- Preclinical tools
- Lessons from other viruses and preparation for the future.

This report summarizes the proceedings of the meeting, including an overview of therapeutic approaches, individual panel summaries, and a summary of the discussions and perspectives on the challenges ahead for antiviral development. Each section below captures discussion points in the corresponding session of the agenda listed in [Box 1](#).

OVERVIEW OF THE VIRUS AND THERAPEUTICS APPROACHES

Coronaviruses are enveloped positive-sense single-stranded RNA viruses enclosed by capsid comprised of multiple proteins, most notably the spike proteins that are responsible for the virus's crown-like appearance. Cellular entry of coronaviruses, such as SARS-CoV-2, occurs primarily via the spike (S) proteins of SARS-CoV-2 binding to the host ACE2 (angiotensin-converting enzyme 2) receptor [1]. Two-thirds of the SARS-CoV-2 genome is dedicated to the synthesis of 2 replicase polyproteins called polyprotein 1a and polyprotein 1ab. Within the polyprotein are 2 proteases, the papain-like protease (PL^{pro}) and 3CL^{pro} (also known as nsp5 or M^{pro}, short for main protease). In general, any inactivation of these proteases leads to a loss of RNA synthesis, and it is well established that viral RNA replication and viral protease functions are vulnerable to intervention [2, 3]. A multiprotein complex formed by coronaviruses termed the replication transcription complex (RTC) is responsible for RNA replication and proofreading. Nonstructural proteins nsp7 through nsp16 form the core of the RTC and represent a prime target for antiviral drug development, as the function of the core replicase is highly conserved. A detailed outline of the viral replication process and druggable targets is displayed in [Figure 1](#).

Importantly in the context of antiviral drugs, when the SARS-CoV-2 epidemic (now a pandemic) emerged, there were no approved treatments or vaccines for treating any coronavirus infection. The endemic common cold betacoronaviruses OC43-CoV and HKU1-CoV generally cause mild symptoms and have not stimulated significant therapeutic or vaccine development investigation, and while NL63 is an alphacoronavirus, it also

uses the ACE2 receptor to mediate infection. The SARS (severe acute respiratory syndrome caused by SARS-CoV) pandemic was resolved using public health measures, but MERS (Middle East respiratory syndrome, caused by MERS-CoV) is still causing sporadic human infections and remains a global threat. The translational science response to the SARS-CoV-2 pandemic has benefited from the intensive research efforts that took place to understand the SARS and MERS CoVs [4, 5].

From a therapeutic perspective, there are multiple windows of opportunity for preventing and managing COVID-19, which include the prophylactic preinfection stage, early postinfection pre- or asymptomatic stage, and the symptomatic stages during which antiviral efficacy wanes and treatment strategy shifts towards immunomodulatory and anticoagulant therapies. However, the window for antiviral intervention remains to be defined fully, especially in later disease stages, because of the lack of availability of a sufficiently potent antiviral agent or combination. As antiviral drugs are developed, some principles for SARS-CoV-2 antiviral development to be considered include:

- Antivirals should be potent against SARS-CoV-2 (and preferably broadly active against coronaviruses).
- Bioavailability of antivirals to sites of infection should be tested for multiple routes of administration: intravenous, intramuscular, subcutaneous, oral, inhalational, intranasal, etc.
- Antivirals should have limited toxicity, especially for outpatient use, preferably have a high barrier to resistance emergence, and improved potency to enlarge the therapeutic window.
- Combinations of antivirals should be a priority to maximize potency and prevent resistance emergence. Combinations of antivirals and immunomodulatory therapies will be needed for treatment in later stages of illness.
- Vulnerable populations should be considered when designing new therapeutics as well as designing clinical trials.

Antiviral drug development for SARS-CoV-2 should take place with the recognition that unlike SARS and MERS (that sporadically reemerges), the pandemic may continue for a long time, and the virus itself will likely become endemic. There is also the high likelihood for another novel coronavirus(-es) to emerge from animal reservoirs or by recombination, and the emergence of variants. Given these realities, irrespective of vaccine effectiveness, there will be a need for antivirals. Teams working together across industry, academia, and government can drive fundamental discovery and create the “playbook” for responding to the next zoonotic coronavirus. In the context of future pandemics, antivirals with activity against other betacoronaviruses would be positioned to enable rapid preclinical testing and clinical trials if and when a new zoonotic coronavirus appears (and for sporadic MERS outbreaks) [6].

Box 1. NIH SARS-CoV-2 Antiviral Therapeutics Summit Agenda 6 November 2020 Virtual Meeting

Purpose

This summit will provide an overview of the current state of direct anticoronaviral targets and therapeutics, available tools, and challenges.

Introductory comments

Dr Francis Collins, Director, National Institutes of Health (NIH)

Dr Anthony Fauci, Director, National Institute of Allergy and Infectious Diseases (NIAID)

Dr Christopher Austin, Director, National Center for Advancing Translational Sciences

Overview of the virus and therapeutics approaches

Dr Mark Denison, Vanderbilt University

Viral Replication Machinery

Moderator: Dr Tomas Cihlar, Gilead Sciences

Dr Elizabeth Campbell, Rockefeller University

Dr Matthias Götze, University of Alberta

Dr George Painter, Emory University

Dr Michael Sofia, Arbutus Biopharma, Inc

Dr Sandra Weller, University of Connecticut

Highlight on status of vaccines and neutralizing antibodies to prevent and treat SARS- CoV-2 infection

Dr Kizzmekia Corbett, Vaccine Research Center, NIAID

Proteases (viral and host)

Moderator: Dr Annaliesa Anderson, Pfizer

LTC Charlotte Lanteri, Walter Reed Army Institute of Research

Dr Andrew Mesecar, Purdue University

Dr Jennifer O. Nwankwo, 1910 Genetics

Dr Celia Schiffer, University of Massachusetts Medical School

Emerging targets, emerging modalities

Moderator: Dr Kara Carter, Evotec

Dr David Baker, University of Washington

Dr Lillian Chiang, Evrys Bio

Dr Matthew Disney, Scripps Research

Dr Kumar Saikatendu, Takeda Pharmaceuticals

Dr Marla Weetall, PTC Therapeutics, Inc

Preclinical Tools

Moderator: Dr Pei-Yong Shi, University of Texas Medical Branch

Dr Sara Cherry, University of Pennsylvania

Dr Emmie de Wit, NIAID/Rocky Mountain Laboratories

Dr Jules O'Rear, US Food and Drug Administration

Dr Timothy Sheahan, University of North Carolina

Dr Hugh Smyth, University of Texas at Austin

Lessons from Other Viruses and Preparation for the Future

Moderator: Dr Daria Hazuda, Merck

Dr Jay Bradner, Novartis

Dr Courtney Fletcher, University of Nebraska Medical Center

Dr Frederick Hayden, University of Virginia

Dr Hilary Marston, NIAID

Summary of discussions and perspectives on the challenges ahead

Dr Richard Whitley, University of Alabama at Birmingham

Dr George Painter, Emory University

Closing statement

Dr Francis Collins, Director, NIH

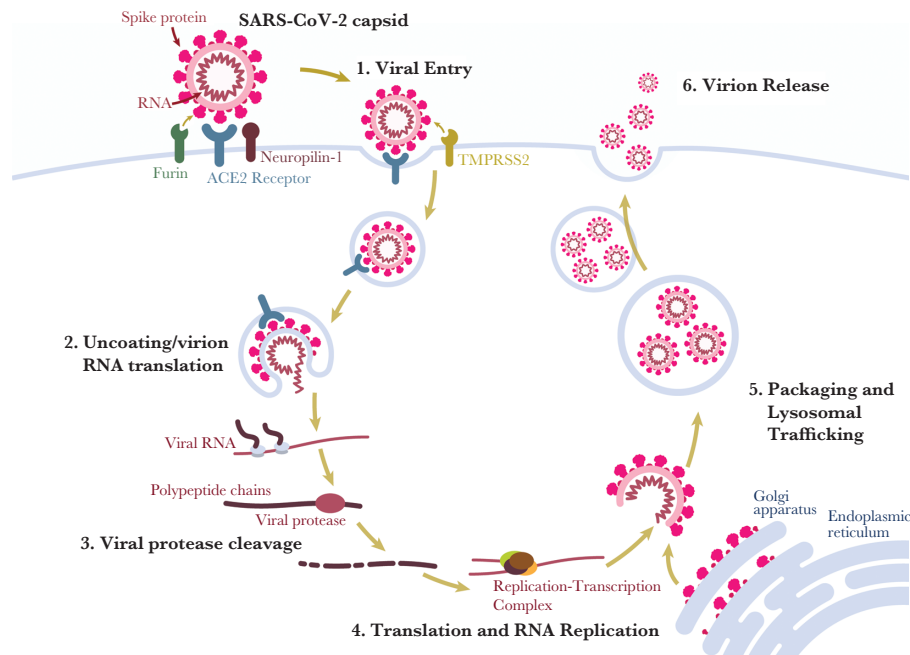


Figure 1. Scheme showing the SARS-CoV-2 viral replication cycle and “druggable” events. The SARS-CoV-2 virion is composed of a capsid protein coat, with an internal core of the viral genetic material (RNA). 1. Viral entry. The SARS-CoV-2 virion in the extracellular space presents Spike (S) protein on the capsid surface. The S protein contains a number of host protease cleavage sites, as well as the receptor binding domain (RBD). Engagement of spike protein at the extracellular surface involves engagement of multiple host cell proteins including (but may not be limited to) cleavage of S by the protease furin (gene *FURIN*), binding of the S RBD to ACE2 (*ACE2*), engagement of a liberated S terminal peptide to neuropilin-1 (*Nrp-1*, *NRP1*), and cleavage of S by the serine protease TMPRSS2 (*TMPRSS2*). 2. Following endocytosis, the virion is uncoated, and the large positive-sense viral RNA translated into polypeptide chains. 3. Two viral proteases cleave the viral polypeptide chains to produce up to 29 mature protein products. These proteases are the main protease (Mpro) and the papain-like protease (PLpro). 4. Viral RNA replication follows, with the formation of a replication-transcription complex, incorporating the RNA-dependent RNA polymerase (RdRp). 5. The arising viral RNA (genetic material) is then packaged into capsid formed by viral protein including envelope (E), membrane (M), nucleocapsid (N) and aforementioned spike. Mature packaged virion is then trafficked via lysosomes, and 6. Virion is released via exocytosis. Schematic prepared by Kyle R. Brimacombe, NCATS. Abbreviations: ACE2, angiotensin-converting enzyme 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

VIRAL REPLICATION MACHINERY

Since the emergence of SARS-CoV-2 at the end of 2019, considerable progress over a very short period of time has been made in the development of antivirals for the treatment of COVID-19, in part because of studies for inhibitors of SARS-CoV and MERS-CoV. The identified agents include small-molecule antivirals and biologics targeting both viral and host functions critical for SARS-CoV-2 infection and replication. Multiple potent neutralizing antibodies targeting independent epitopes on viral spike protein alongside a battery of direct-acting small-molecule antivirals interfering with various steps of viral replication have been quickly identified and brought to clinical testing. Across these efforts, viral RNA synthesis stands out as one of the most critical functions for selective targeting by antiviral therapies [7].

SARS-CoV-2 and other coronaviruses contain an approximately 30-kb positive-sense nonsegmented single-strand RNA genome. Their RNA synthesis is a complex process consisting of 2 independent parts:

1. Viral genomic RNA replication through synthesis of a full-length negative (–) strand copy of the viral RNA genome that

serves as a template for subsequent amplification of the genomic positive strand (+) RNA.

2. RNA transcription progressing via synthesis of subgenomic (sg) (–)RNA intermediates that are subsequently transcribed into mRNA. Some of the (–)sgRNA species are made through discontinuous processes that include template switching. Once synthesized, the viral mRNAs are 5'-capped and 3'-polyadenylated to enable efficient translation of viral proteins.

All steps of the coronavirus RNA synthesis are carried out by the viral RTC encoded by approximately one-third of the viral genome [8, 9]. The RTC is an assembly of at least 9 viral nonstructural proteins (nsp) known as nsp7 through nsp16, as well as some less characterized host factors. Core catalytic function of the RNA synthesis is performed by RNA-dependent RNA polymerase (RdRp; nsp12) and 2 of its cofactors (nsp7 and 8). Coronaviruses also encode for RNA proofreading 3'-5' exonuclease function, carried out by nsp14, that increases the fidelity of replication and maintains genetic stability of the large coronavirus genome. The 5'-capping of RNA is performed by 2 methyltransferases (nps14 and nsp16). Table 1 summarizes the viral RTC proteins and their functions.

Viral nucleic acid synthesis has been successfully targeted by a wide range of small-molecule direct-acting antivirals, many of which have been developed into commercial drug products and are widely used in clinical practice [10]. Examples include inhibitors of human immunodeficiency virus (HIV) reverse transcriptase (a DNA polymerase), hepatitis C virus (HCV RNA polymerase), and hepatitis B virus (HBV) and herpesvirus DNA polymerases. While some of these enzymes can be targeted effectively by non-nucleoside allosteric inhibitors (eg, non-nucleoside reverse transcriptase inhibitors for HIV), nucleoside and nucleotide analogs mimicking the natural nucleic acid building blocks remain the most abundant class of viral replication inhibitors and became the backbone of a number of combination curative therapies [11]. It should be noted that these examples are all treating chronic infections, whereas SARS-CoV-2 replication peaks prior to or early in disease symptoms, resulting in an extremely limited treatment window.

Antiviral inhibitors of coronaviruses, including SARS-CoV-2, have been identified among nucleoside and nucleotide analogs with known antiviral activity against other RNA viruses, several of which quickly progressed into clinical testing for the treatment of COVID-19 (Figure 2). Table 2 lists a summary of profiles of these agents. Each candidate is a prodrug that requires intracellular activation to a triphosphate form that is incorporated into viral RNA, resulting in effects including delayed RNA termination, RNA mutagenesis, and/or second RNA strand synthesis stalling.

Remdesivir is a phosphoramidate prodrug that liberates an adenosine nucleotide analog within cells (the core nucleoside being GS-441524) [12]. Remdesivir was developed by Gilead Sciences with support from several US Government organizations. Remdesivir was shown to be active against Ebola virus, and its safety profile was demonstrated in Ebola clinical trials. In several randomized clinical trials, remdesivir has demonstrated efficacy in hospitalized COVID-19 patients by reducing disease progression and accelerating time to recovery [13]. In October

2020, remdesivir was approved by Food and Drug Administration (FDA) as the first treatment for COVID-19 [13, 14].

Molnupiravir (EIDD-2801) is an orally bioavailable prodrug of N6-hydroxycytidine. It was developed at Emory (University) Institute for Drug Discovery (EIDD) as part of a program against Venezuelan equine encephalitis virus, and preclinical models have shown antiviral activity against both influenza and respiratory syncytial viruses. A phase 1 trial was planned against influenza in 2019 [15]. As SARS-CoV-2 emerged, molnupiravir showed potent anti-SARS-CoV-2 activity both in vitro and in animal models [16]. It is currently in phase 2–3 testing both in outpatient settings and in hospitalized COVID-19 patients.

Favipiravir, a guanine base analog, is a drug product approved in Japan (2014) for the treatment of influenza infections due to novel strains not responsive to other available agents [17]. Phase 3 trials in uncomplicated influenza demonstrated antiviral but variable clinical efficacy, and favipiravir is more active in vitro for influenza viruses than SARS-CoV-2 [18]. It is also under regulatory review for the treatment of COVID-19 in Japan and has recently been approved for the same indication in Russia. It is active against SARS-CoV-2 in the hamster model and is being tested in several advanced clinical studies around the world [19]. While it can be administered orally, it is substantially less potent than molnupiravir and remdesivir in vitro, which is reflected in a much higher dose required for favipiravir.

AT-527 is a prodrug of a guanosine nucleotide analog, developed by Atea Pharmaceuticals [20]. AT-527 was developed against the HCV RdRp, and safety was demonstrated in an HCV phase 1–2 clinical trials in healthy and HCV-infected subjects. AT-527 was recently found to be active in vitro against SARS-CoV-2, and is currently being tested in phase 2 in patients hospitalized with moderate COVID-19 disease [21].

Galidesivir is an adenine nucleoside analog developed by BioCryst for HCV, but it has activity against other RNA viruses and has been in development for treating filoviruses (eg, Ebola, Marburg) [22]. It has shown relatively weak activity in vitro against SARS- and MERS-CoVs and is slated for testing in a small proof-of-concept phase 2 study in Brazil. It is the least characterized molecule among the SARS-CoV-2 nucleoside inhibitors.

It is worth noting that all the drug candidates described above existed before the COVID-19 pandemic, and novel chemical matter developed specifically for the SARS-CoV-2 replication machinery has not yet been disclosed. While diverse in structures as well as formulations administered (Figure 1 and Table 2), all SARS-CoV-2 replication inhibitors require intracellular activation to their nucleoside triphosphate (NTP) metabolites, which then act as competitive alternative substrates of viral RdRp and are incorporated into viral RNA. The metabolic pathways leading to the respective NTP metabolites are cell-type dependent and differ for each molecule, as do their molecular mechanisms of viral RNA synthesis inhibition. Once

Table 1. Virally Encoded Components of Coronavirus RTC

Coronavirus Protein	Function in RTC
nsp7	RdRp cofactor
nsp8	RdRp cofactor
nsp9	RNA binding protein
nsp10	Cofactor of nsp14 and nsp16
nsp12	RNA-dependent RNA polymerase, capping
nsp13	Zn-binding RNA helicase/RNA 5'-phosphatase
nsp14	3'-5' exonuclease; N7-methyltransferase
nsp15	Uridylate-specific endoribonuclease
nsp16	2'-O-methyltransferase

Abbreviations: nsp, nonstructural protein; RdRp, RNA-dependent RNA polymerase; RTC, replication transcription complex.

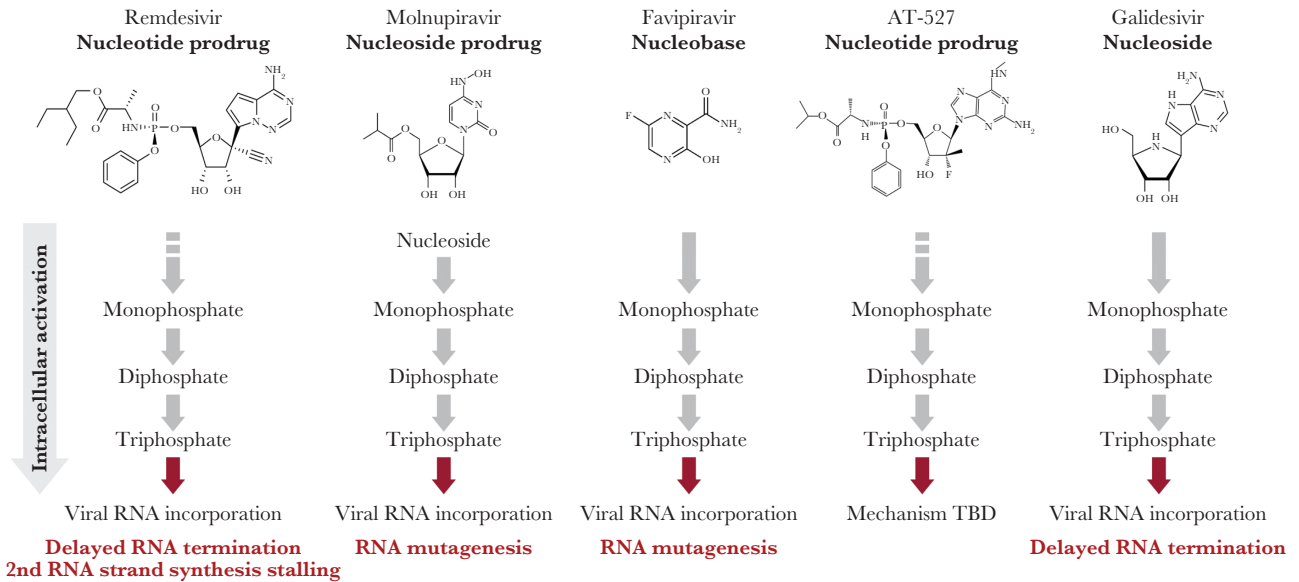


Figure 2. Structures of nucleoside analogs for the treatment of COVID-19 and their metabolic activation. Abbreviations: COVID-19, coronavirus disease 2019; TBD, to be disclosed.

incorporated into viral RNA, the metabolites of remdesivir and galidesivir cause delayed RNA chain termination [23, 24]. In the case of remdesivir template-dependent inhibition has also been shown, while those of molnupiravir and favipiravir act as specific viral RNA mutagens due to their capability of promiscuous base pairing [25]. The most substantive, yet minor, concern was the suggestion that the mechanism of action of favipiravir is lethal mutagenesis. Evidence is accumulating that an alternative mechanism unrelated to mutagenesis may be operative with pyrazine carboxamides [26]. The mechanism of action for AT-527 is less well characterized, and structural as well as biochemical data suggest that it might not interact very effectively with SARS-CoV-2 RdRp.

Challenges and Opportunities

Nucleoside and nucleotide analogs represent the most extensively explored class of antivirals with well understood advantages and limitations that both are related primarily to their function as mimetics of natural nucleotides. Because the active sites of viral RNA polymerases to which the active NTP metabolites bind are often highly conserved within as well as across multiple viral families, many nucleosides exhibit antiviral activity against other RNA viruses. Remdesivir, favipiravir, and molnupiravir are good examples of broad-spectrum antivirals with activity against RNA viruses that may be leveraged as a part of future pandemic preparedness not only for the treatment of COVID-19, but likely also for other existing and newly emerging coronaviruses, and in the case of favipiravir and molnupiravir for novel influenza viruses.

The similarity of antiviral nucleoside analogs with natural substrates can also represent a significant liability, mainly because

of their potential for off-target effects such as impairment of mitochondrial functions through the inhibition of (host) mitochondrial RNA polymerase. Some nucleoside analogs might require prodrug strategies using established principles of medicinal chemistry to optimize oral delivery (bioavailability). In the case of SARS-CoV-2, the primary target tissue is the lung, and effective distribution into target cells irrespective of administration route is required.

Besides the inhibition of nsp12 (RdRp) by nucleoside analogs, the highly dynamic and multifunctional coronavirus RTC offers variety of other opportunities to interfere with viral RNA synthesis, including the other critical catalytic enzymatic functions present in RTC such as the nsp13 helicase, nsp14 and nsp16 methyltransferases, or nsp14 exonuclease. In addition, the concerted function of this multiprotein complex relies on a multitude of specific protein-protein interactions, many of which have been mapped across the spectrum of coronaviruses including SARS-CoV-2. These efforts can be greatly facilitated by structural information generated by X-ray crystallography and cryoelectron microscopy studies conducted with many of the RTC proteins and their complexes, including the nsp15 endonuclease, nsp14/10 and nsp16/10 methyltransferases, and particularly the multiprotein RdRp complex of nsp12/nsp7/nsp8 together with a dimer of nsp13 helicase. This wealth of structural information can facilitate the identification of critical interfaces and conserved pockets that might be amenable to targeting with small-molecule inhibitors.

These alternative RTC targets offer the opportunity for non-nucleoside drug-like molecules, with the potential for candidates with desirable pharmacokinetic properties, like oral bioavailability, low protein binding, and adequate delivery to

Table 2. Nucleoside Analogs for the Treatment of COVID-19; Status as of November 2020

Nucleoside Drug	Form	Mechanism of Action	In Vitro Activity, EC ₅₀ , μ M	Animal Model Activity	Clinical Dosing	Development Status (Target Population)
Remdesivir (GS-5734)	Nucleoside monophosphate prodrug	Delayed RNA chain termination	0.01–1.5 [70, 74]	NHP [92], mouse [74]	IV once daily 200/100 mg	Approved in United States and 50+ countries (hospitalized); phase 3 (outpatient)
Molnupiravir (EIDD-2801, MK-4482)	Nucleoside prodrug	Viral RNA mutagenesis	0.05–0.3 [16]	Mouse [16]	Oral twice a day 200–800 mg	Phase 2/3 (outpatient and hospitalized)
Favipiravir (F-705)	Nucleobase	Viral RNA mutagenesis	60–200 [19]	Hamster [19]	Oral twice a day 1800/800 mg	Approved in Russia; under review in Japan
AT-527	Nucleoside monophosphate prodrug	TBD	0.5 (EC ₅₀) [21]	TBD	Oral twice a day 550 mg	Phase 2 (hospitalized)
Galidesivir (BCX-4430)	Nucleoside	Delayed RNA chain termination	58–68 ^a [23]	TBD	IV	Phase 1b

Abbreviations: EC₅₀, 50% effective concentration; IV, intravenous; NHP, nonhuman primate; TBD, to be disclosed.

^aIn vitro activity against SARS-CoV and MERS-CoV.

the respiratory tract. Unfortunately, the current state of the field underscores a complete absence of any validated small-molecule inhibitors targeting some of these critical replicative functions, which in turn hinders advancement towards optimization of any new class of RTC inhibitors and their rapid progression into clinical testing. Therefore, the drug discovery process will rely on de novo high-throughput screening approaches of suitable small-molecule libraries. To that end, a number of recombinant RTC proteins have been cloned, expressed, and functionally characterized. Several options for functional biochemical assays exist depending on the desired format and preferred mode of read-out, particularly for the catalytic complex of nsp12/nsp7/nsp8 RdRp. Similarly, functional assays have been established for coronavirus exonuclease as well as methyltransferases.

To effectively target some of the critical protein-protein interfaces, the field can employ a wide array of screening techniques for small-molecule ligand binding, some of which are quite suitable for large compound libraries. Options include high-throughput affinity selection mass spectrometry, DNA-encoded libraries, or surface plasmon resonance assays. Alternatively, computational docking algorithms for putative binding sites can be employed for screening of vast virtual small-molecule libraries. The major advantage of these approaches is speed and the number of potential candidate molecules identified, but these techniques need to be always coupled with relevant functional testing assays to triage the hits and validate their functional relevance. Newly developed cell-based replicon assays for SARS-CoV-2 should be valuable to characterize activity of candidate compounds and certify cell-based activity and activation of candidate prodrugs [27, 28].

Work remains to identify optimal target combinations with RdRp/RTC inhibitors, both small-molecule antivirals and other modalities/approaches, and the patient populations most likely to benefit depending on individual drug product profiles, to maximize clinical effectiveness in the treatment and/or prevention of COVID-19.

PROTEASES (VIRAL AND HOST)

The SARS-CoV-2 Replication Cycle Requires Host and Viral Proteases

SARS-CoV-2 uses both host and viral proteases in its replication cycle (Figure 1). The generalizability of host proteases makes them a good target for many distinct coronaviruses that tend to use similar host factors. The spike proteins of SARS-CoV and SARS-CoV-2 have commonalities, such as sequence similarities and multibasic proteolytic cleavage sites at both the S1 and S2 residue site as well as the S2 prime residue sites where host proteases (such as furin and TMPRSS2) cleave to facilitate viral entry. The risk of side effects is higher when targeting host functions and, contrary to dogma, viruses can develop resistance to drugs targeting host functions [29–31].

Host proteases TMPRSS2 and furin have a role in cleaving spike protein between S1 and S2 to reveal the fusion peptide

and helical repeat domains that are necessary for viral entry [32, 33]. Currently, 2 compounds (camostat and nafamostat) are being investigated that potentially inhibit the host proteases and could be used as COVID-19 treatments. Both are repurposed drugs that are approved in Japan for the treatment of several conditions, including pancreatitis [34]. Because these compounds have existing human safety data, late-stage clinical studies for COVID-19 treatment are underway.

Viral Protease Inhibitors Have Been Used to Treat HIV and HCV Successfully

The first viral protease inhibitors were identified in the search for HIV therapies [35]. The HIV protease inhibitors are some of the first examples of structure-based design, a move away from the traditional high-throughput screening approaches that had been previously deployed for drug discovery efforts. A similar approach was also used for HCV and was so successful it was able to provide a cure for what was previously a chronic disease.

Structural design was first applied to coronaviruses in 2003 during the SARS epidemic caused by SARS-CoV, with the discovery of the first protease inhibitors specifically designed for coronaviruses [36]. Both the public health measures that led to control of SARS-CoV and the resulting lack of patients for clinical studies meant that these investigational drugs were not evaluated in clinical trials. It is not yet known if the previously identified SARS-CoV protease inhibitors will be efficacious at treating SARS-CoV-2 infections, although there are encouraging preclinical *in vivo* data on these compounds against SARS-CoV-2 and other coronaviruses with similar proteases.

Two SARS-CoV-2 Cysteine Proteases Can Be Targeted for Potential COVID-19 Treatment

There are 2 SARS-CoV-2–encoded cysteine proteases that are potential antiviral targets: a papain-like protease and a 3C-like protease. These proteases are responsible for cleaving the polypeptide that is translated after the viral RNA enters the host cell. The single polypeptide makes up the machinery that the virus needs for replication. If it is not cleaved into the 16 individual nonstructural proteins, the virus cannot replicate. The papain-like protease is a multifunctional enzyme that is also associated with pore formation and immune modulation [37]. The 3C-like protease, which is also known as the main protease, functions as a dimer and has a single role, which is to cleave the polypeptide at 11 sites [38].

HCV and HIV Drugs Have Limited Utility for COVID-19 Treatment; Current Potential Antiviral Inhibitor Candidates

Early in the pandemic, steps were taken to investigate whether existing HIV and HCV protease inhibitors could be redeployed to treat COVID-19 patients [39]. There are considerable

differences in the structure of the different proteases, so this approach led to little success, although some inhibition was observed. Clinical studies were in progress with the HIV drug ritonavir-boosted lopinavir, but an early controlled trial in COVID-19 patients in China failed to find evidence of antiviral effects or clinical benefits, and subsequent large trials have confirmed the lack of therapeutic efficacy in hospitalized COVID-19 patients [40, 41]. Lopinavir and isotretinoin are examples of drugs repurposed for potential antiviral therapy, although mechanism of the drug's action is unclear. Another protease inhibitor, PF-07304814 (termed PF-814), was specifically designed to inhibit coronavirus proteases, and phase 1 studies are currently ongoing. This compound is administered to hospitalized patients via intravenous infusion. Several protease inhibitors are also under investigation that can be provided as oral therapies to nonhospitalized populations [42].

Example of 3CL Protease Inhibitor Development: Pivoting to a Prodrug Candidate PF-007304814 to Enable Clinical Dosing of Therapeutic Concentrations of Active Drug Candidate PF-0835231

In January 2020, Pfizer restarted its coronavirus protease inhibitor development program that had been conducted in response to the SARS epidemic more than 15 years earlier [36]. After comparing sequences of the 2 coronaviruses, a high degree of amino acid sequence identity was observed between the 3CL proteases, and active site modelling studies indicated that Pfizer's lead compound PF-0835231 (termed PF-231) could potentially be developed and then, if approved, be deployed against SARS-CoV-2.

The following months saw the usual long development program dramatically compressed. This was done in part with the help of prominent coronavirus experts in the field who accumulated evidence that the candidate drug was highly potent and specific for the inhibition of coronavirus 3CL proteases, including that from SARS-CoV-2. When it was recognized that the lead compound's potential pharmacokinetic exposure was suboptimal, a phosphate prodrug candidate termed PF-07304814 (or PF-814 for short) was prepared [43]. This prodrug candidate had increased exposures to allow for effective intravenous dosing in clinical studies, the first of which was initiated in September 2020 (Clinical Trials Registration NCT04535167).

PF-0835231 SARS-CoV-2 Protease Inhibitor Has Additive Activity With Remdesivir *In Vitro* Due to Differentiated Mechanism of Action and Has the Potential to be Broadly Cross-Reactive Across Coronaviruses

Remdesivir and PF-231 have distinct antiviral mechanisms of action, presenting an opportunity for combination therapy. PF-231 demonstrated potent single-agent EC_{90} value in a SARS-CoV-2 *in vitro* antiviral assay with minimal cytotoxicity, indicating potential clinical efficacy for viral protease inhibitors [44]. Combining PF-231 with remdesivir increased

the observed in vitro potency. These in vitro data suggest that a combination may be an effective approach to treat the virus with reduced amounts of each compound.

Collectively, these in vitro data suggest PF-814 (the prodrug of PF-231) has the potential to be effective as a single or a combination agent against SARS-CoV-2. In addition, it appears the compound has the potential to be active against other coronaviruses and could potentially be deployed in the event of future coronaviruses that might emerge as a result of additional species crossover events. Two clinical studies are ongoing: one is a phase 1 single ascending dose study in healthy volunteers (Clinical Trials Registration NCT04627532), and the second is a phase 1b study in patients with COVID-19 (Clinical Trials Registration NCT04535167). A pivotal phase 2/3 study is planned to start in 2021.

PL^{pro} Has Other Functions in Addition to Protease Activity That PL^{pro} Inhibitors May Also Prevent

Coronavirus PL^{pro} inhibitor development is not as advanced as the 3CL^{pro} inhibitors. Inhibitors targeted to this protease may provide a unique opportunity to not only inhibit viral replication via inhibition of cleaving the nsp3, but also inhibit nsp3 from antagonizing the nuclear factor- κ B (NF- κ B) and interferon regulatory factor 3 (IRF3) host innate pathways via deubiquitination and de-ISGylation, as well as removing interferon-stimulated gene 15 (ISG15) from proteins in the Jak/Stat pathway. The dysregulation of these pathways may contribute to excessive cytokine release from infected cells. In addition, nsp3 has recently been shown to be part of a molecular pore in double-membrane vesicles (DMVs). The DMVs form after viral infection as a result of nsp3, nsp4, and nsp6 embedding in the rough endoplasmic reticulum and reshaping it.

Challenges and Opportunities

The challenges and opportunities for the discovery and development of SARS-CoV-2 protease inhibitor treatments have focused on how advanced screening methodologies are being used to accelerate screening and the importance of structural analysis to identify potential resistance challenges and facilitate compound discovery. Current research into protease inhibitors has been enhanced by consortia and collaborations. For example, work conducted by the Center for Structural Genomics of Infectious Diseases uses artificial intelligence approaches to rapidly accelerate drug screening processes and reduce the number of compounds for more involved validation assessments.

With each new mechanistic insight come new targets, including host factors. These targets provide an opportunity for rational drug design accelerated by artificial intelligence (AI) that can be deployed at all stages of the early drug discovery process, from novel hit discovery to identification and optimization. For example, 1910 Genetics, a biotechnology company that integrates AI, computation, and biologic automation in early stage drug discovery, deployed its SUEDE platform, which

screened a 1-billion virtual chemical library in a matter of hours and identified promising virtual candidates against TMPRSS2. Within 2 weeks, these small molecules were in 1910 Genetics' facilities. They were tested in a short period of time, discovering potent analogs (50% effective concentration [EC₅₀] = 7 nM) to block the entry of the pseudotyped SARS-CoV-2 spike protein into Vero6E cells that were engineered to overexpress TMPRSS2. In parallel, 1910 Genetics deployed its BAGEL platform, which generated de novo small-molecule candidates that were manufactured within weeks and tested in 1910 Genetics' in-house facilities. One of these de novo molecules also proved to be potent (EC₅₀ = 117 nM) in blocking the entry of pseudotyped SARS-CoV-2 spike protein into Vero6E-TMPRSS2 cells.

With the arrival of the COVID-19 pandemic, scientists at the Walter Reed Army Institute of Research (WRAIR) initiated a discovery program. This involved a significant pivot for the WRAIR Experimental Therapeutics Branch, the Department of Defense's (DoD's) sole drug development group, into a new therapeutic area of developing antivirals. The WRAIR Experimental Therapeutics team leveraged their inherent drug discovery and development capabilities and expertise for developing malaria and antibacterial drugs to address COVID-19.

WRAIR partnered with US Army Medical Research Institute of Infectious Diseases (USAMRIID) scientists who established a high-throughput in vitro SARS-CoV-2 Vero cell-based test system at their biosafety level-3 (BSL-3) laboratories. As part of this DoD COVID-19 Small Molecule Therapeutics effort, collaborators at the Southwest Research Institute in San Antonio, TX, in conjunction with the DoD's High Performance Computing Modernization Center, applied a proprietary AI/machine learning algorithm to screen >41 million compounds against 2 SARS-CoV-2 targets: the main protease and spike protein receptor domain. The virtual screens were based on published crystal and cryoelectron structures, as well as X-ray structures of the virus produced by the WRAIR Emerging Infectious Diseases Branch. The DoD group screened a library of >41 million compound structures in a matter of weeks. Compound structures came from a diversity of sources; some of them were existing drugs that may be repurposed for SARS-CoV-2 and other coronaviruses. Others were novel chemical matter from a large repository of compounds that had already been analyzed for drug-like properties. In contrast to traditional in silico screening, this method introduces the ligand to the target with no preconceived notions of binding pose. Their ultimate goal is to develop a pan-coronavirus drug; as such, virtual screen hits were also screened against an analogous target (the receptor binding domain) from SARS-CoV and MERS to select compounds with the greatest overlap in predicted binding. From this large-scale initial screen, they rapidly down-selected approximately 800 compounds within a few weeks, dramatically shortening the traditional years-long discovery phase. To date, testing of in silico hits resulted in a 9% hit rate in USAMRIID's

SARS-CoV-2 antiviral Vero cell screen. This outcome is orders of magnitude better than more traditional high-throughput methods. The combination of AI and then high-throughput screening using an in vitro assay has been key for accelerating the discovery of potential new coronavirus treatments. The molecules are currently being studied from a medicinal chemistry perspective, including determination of their metabolic properties, safety evaluation, and pharmacokinetics in mouse models in preparation for murine SARS-CoV-2 efficacy testing.

PL^{pro} Development Progress

Dr Mesecar of Purdue University described working on PL^{pro} and M^{pro} after the SARS-CoV-1 pandemic in 2003–2004 with a collaborative team [45–48]. At that time, the papain-like protease was an overlooked protease with no established structure. Cleavage of ubiquitin off host cell proteins was also identified as another potential role for the PL^{pro}. Dr Mesecar and his group confirmed that PL^{pro} was capable of utilizing ubiquitin substrates. He evaluated ISG15 and determined it is also capable of hydrolyzing 7-amino-4-methylcoumarin of ISG15, causing its removal from ubiquitin in host cells. Understanding the complete role of PL^{pro} antagonism in an aspect of the host innate immune response will be important to the successful use of inhibitors of this protease.

Dr Mesecar and colleagues elucidated the structure of PL^{pro} and found that it was indeed a ubiquitin-specific protease. They then conducted a high-throughput screen of 50 000 compounds, identifying 2 potential compound templates for antiviral drug development. These noncovalent inhibitors had the ability to target only a certain subclass of coronaviral papain-like proteases selectively, making it possible to target papain-like proteases selectively. To date, these compounds have shown activity in a non-SARS-CoV-2 coronavirus animal model.

Design of Protease Inhibitors to Preemptively Avoid Drug Resistance

Viral proteases are key drug targets and have been one of the most successful structure-based drug designs. However, drug resistance can come from mutations in the active site or remote changes that alter the flexibility of the protease [49–51]. The necessity of the protease to cut a series of cleavage sites is a key evolutionary constraint. Dr Celia Schiffer's laboratory at University of Massachusetts Medical School, for example, has focused on this evolutionary constraint of substrate recognition and processing and how to combine that evolutionary constraint to preemptively avoid drug resistance. Inhibitors that fit within the substrate envelope are less likely to produce resistant strains because the mutation impacting these inhibitors will simultaneously impact the recognition and processing of the majority of the substrates. Dr Schiffer's team has previously demonstrated this approach with HIV and HCV proteases [52–54]. Similar to other viruses, SARS-CoV-2 has already evolved during the current pandemic. As the SARS-CoV-2 viral proteases both

recognize multiple substrates, the substrate envelope approach is being pursued as a way to preemptively avoid resistance. Combining protein crystallography, medicinal chemistry, and computational methods to SARS-CoV-2 3CL^{pro} and PL^{pro} with a collaborative team at University of Massachusetts Medical School, the aim is not only to have inhibitors that retain effectiveness during the current pandemic but are potent against any future coronavirus outbreaks.

EMERGING TARGETS, EMERGING MODALITIES

As discussed above, small-molecule inhibitors of essential viral enzymes, such as polymerases and proteases, are well-validated approaches for antivirals. However, as with all complex viral diseases, multiple therapeutics to address different aspects of the SARS-CoV-2 viral replication cycle and in different COVID-19 patient populations are needed to more effectively treat patients.

Several new modalities have arisen for therapeutics in general over the last several years, including RNAi, CRISPR/Cas9 and other targeted nucleases, bifunctional molecules, and others. Dr Matt Disney of Scripps Research Institute described how sections of RNA virus genomes in general, and SARS-CoV-2 in particular, form constrained secondary structures that are specifically targetable by small molecules. Such regions of the SARS-CoV-2 genome include the 5' untranslated region and the frame shift motif. Small-molecule screens have been conducted and identified binders to these elements [55]. These binders have been used as the basis of a chimeric compound that can target a host ribonuclease to the viral genome and ultimately lead to its degradation. Such a mechanism has demonstrated potent antiviral activity. In addition, Dr David Baker of the University of Washington described his work generating miniproteins that specifically bind to the receptor binding domain of the viral spike protein with potent nanomolar antiviral activity [56]. These compact, thermostable proteins were computationally designed and are able to be produced in large quantities that could lead to minimal cost of goods. When fused to an antibody Fc domain, the lead SARS-CoV-2 antiviral miniprotein reduces viral burden, diminishes immune cell infiltration and inflammation, and completely prevents lung disease and pathology in mice. A single nasal dose also reduced infection in the lung even when given as many as 5 days before or 2 days after virus inoculation. Importantly, the protein was demonstrated to protect animals against a historical strain (WA1/2020), an emerging B.1.1.7 variant, and a variant encoding key E484K and N501Y spike protein mutations [57]. These data support development of such antiviral miniproteins for prevention or treatment of COVID-19.

Both of these newer modalities have potential advantages. First, drug discovery can begin as soon as a new genome is sequenced (a parallel with vaccine development). For miniproteins, computational methods lead to the synthesis

of small numbers of molecules for testing. For RNA binders, analysis and comparison to sequences of related viruses can accurately predict genome sequences/structures to start screening for small-molecule binders. The miniproteins, as mentioned above, can be developed with different formulations for different routes of administration and have good potential for low cost. Additionally, at least in the case of the miniprotein generated against SARS-CoV-2 spike, the molecule is thermostable and can be stored at room temperature. The RNA-targeting small molecules may have a lower incidence of mutation given there are only 3 options of nucleotides to be incorporated into the RNA for such mutations, and to maintain functional structure of the RNA, even substitution by those 3 options may not be tolerated by the virus. Both of these modalities, however, lead to molecules that will likely have activity limited to viruses with close sequence homology in the viral target.

Complementing the advances in new modalities is the identification of novel targets for antivirals beyond the traditional viral enzymes such as polymerases and proteases. Both systems biology studies [24, 58] and compound repurposing screens [45, 59–63] have identified a number of potential host cell targets to be explored for validation and ultimately drug discovery and development. One example is dihydroorotate dehydrogenase (DHODH), an essential host enzyme in *de novo* pyrimidine biosynthesis. Studies to identify pathways and/or targets can vary in their methodology, including use of different cell types and tagging or knock-out of proteins, which can lead to conflicting results. The public visibility and combination of large data sets may allow identification of potential targets. These targets become actionable as they are verified with the use of probe molecules to test the therapeutic hypothesis, and demonstration of activity in multiple orthogonal assay systems.

Drug discovery focused on host cell targets is anticipated to present multiple opportunities that will require validation. As opposed to direct-acting antivirals with a more defined developmental path, drugging host proteins or processes may present less potential for resistance emergence and should be effective irrespective of the viral load. Additionally, such molecules tend to be more refractory to drug block release, meaning that after removal of the drug, the changes that the drug induced in the host cell are maintained by extending the antiviral effect. Particularly compelling cellular targets are those engaged in stress-activated signal transduction and the dysregulated metabolism induced upon viral infection [64, 65]. For example, Evrys Bio, LLC is developing inhibitors of host SIRT2, which have demonstrated potent and durable antiviral activity across a number of viruses [66, 67]. Cellular receptors for viral entry are also a viable target, exemplified by CCR5 inhibitors approved for treatment of HIV infection, and studies are ongoing to develop (protein-based) ACE2 decoys that limit SARS-CoV-2 cellular entry [68, 69].

As host genes are explored as targets for SARS-CoV-2 drug discovery, rigorous validation requires focus on the biology of the target. Additionally, as new modalities are explored, an emphasis on physical properties, pharmacokinetics, formulation, and other key pillars of drug development are needed. These new targets and modalities do not replace small-molecule drug discovery against traditional, validated targets like polymerases and proteases. Rather, they are expected to complement those efforts and increase the diversity of agents that can be used to treat COVID-19 patients. In some cases, these approaches may provide more rapid discovery and development of drugs in a pandemic setting. The breadth of activity and potency, even in the face of high viral load, of drugs targeting cellular proteins can complement more traditional antivirals. These new targets and modalities should certainly be explored as part of the COVID-19 response.

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PRECLINICAL TOOLS

Biologically and clinically relevant models are required for the evaluation of antiviral drug candidates to obtain the most meaningful and informative preclinical data. Both *in vitro* (cell-based) and *in vivo* (animal) models of SARS-CoV-2 infection have been developed to evaluate antiviral activity and therapeutic activity of candidate drugs. Various *in vitro* systems have been used to identify potential COVID-19 therapeutics in the screening of drug libraries comprised of approved drugs with the hope of repurposing them to treat COVID-19 and by screening of compound libraries to identify new antiviral compounds to then be optimized into a more drug-like form through medicinal chemistry. Animal models of SARS-CoV-2 infection that recapitulate viral tropism and COVID-19 disease pathology are required to ensure that preclinical candidates are active at the target site (eg, lung) and to assess antiviral combinations. As SARS-CoV-2 is a respiratory virus, formulation of oral or possibly inhaled delivery of therapeutics will be most impactful as dosing would not require assistance from a medical professional. Preclinical animal models are essential to determine if therapeutic candidates have antiviral activity, optimal pharmacokinetic profiles, and mitigate SARS-CoV-2 pathogenesis.

In Vitro Models

For cell-based antiviral testing, both wild-type virus and those engineered to express reporter genes like luciferase or fluorescent proteins have been used to screen compounds for antiviral

activity [70, 71]. A single-round infectious SARS-CoV-2 that recapitulates authentic virus infection and replication without virulence has recently been developed to facilitate antiviral screening at BSL-2 [72]. Antiviral activity and potency have been determined by multiple techniques, including through the measurement of reporter gene products, direct measure of infectious virus by classical virologic techniques, measurement of viral cytopathic effect in cell monolayers, and immunostaining-based measurement of viral proteins via high-content imaging, which can also monitor cytotoxicity [59]. To most efficiently triage compounds, early removal of toxic compounds is preferable. Compared with wild-type virus, reporter virus assays typically have a higher dynamic range and potentially higher throughput capacity. High-throughput assay formats also enable the screening and assessment of antiviral combinations in cell culture, which require a large number of experimental conditions to be evaluated [73].

The target cell type employed is critical to accurately predict antiviral activity of candidates in humans. A number of seemingly simple considerations, including the species of origin of a given cell line (eg, human versus other animal-derived cell lines), and the tissue of origin (eg, respiratory epithelial versus kidney or colon) significantly impact the relevance of antiviral candidate potency. For example, remdesivir EC_{50} values differed by 1000-fold when remdesivir was tested in Vero cells (African green monkey kidney epithelial cell line, weak activity) compared with primary human airway culture (potent activity) [74]. This is likely due to differences in cellular enzymes that transport and/or metabolize the prodrug into its pharmacologically active triphosphate form. Conversely, chloroquine and its derivatives showed anti-SARS-CoV-2 activity in Vero cells but not on primary human airway cultures or animals [75], and subsequent clinical trials have confirmed that hydroxychloroquine is ineffective for prophylaxis or treatment of SARS-CoV-2 infections in humans [40, 41, 76]. These results underline the importance of selecting the right cell types for antiviral testing, and primary or multicellular airway models should be used to confirm antiviral activity.

Reverse Genetic and Other Target Identification Systems

Reverse genetic systems have been developed for SARS-CoV-2, leading to the generation of both fluorescent and luminescent reporter viruses that can increase the throughput of screening assays with authentic virus particles at BSL-3 [77, 78]. In addition, reverse genetics enables the phenotypic confirmation of drug resistance mutations derived through passage in cell culture or those that naturally arise in humans and drug target deconvolution in mechanism-of-action studies with newly discovered drug candidates. Alternatively, functional genomics screening approaches such as CRISPRi can enable the identification of human (host) genes that play a cooperative role in mediation of the viral replication cycle and may be druggable.

In Vivo Models

Several animal models have been developed for SARS-CoV-2, including mouse, hamster, ferret, and nonhuman primates. A detailed list of SARS-CoV-2 animal models is maintained by the NIH ACTIV Preclinical Working Group at the NCATS OpenData Portal [79, 80]. For both small animal and nonhuman primate models, information on background, primary references, and viral model end points are provided. The antiviral activity (EC_{50} value) of candidate agents should be evaluated against the challenge virus to ensure that the susceptibility is representative of circulating strains.

The establishment of any animal model relies on that species being susceptible to infection by SARS-CoV-2 [81], and a secondary consideration is the species being amenable to maintenance in a vivarium setting. Although mice are typically used widely to study viral pathogenesis and antiviral efficacy due to their size, ease of use, available genetic models, and experimental tools, due to differences in the murine ortholog of the human receptor, ACE2, the original strains of SARS-CoV-2 spike cannot bind mouse ACE2 and cannot infect standard laboratory mice. This has changed with the emergence of the N501Y variant strain (such as B.1.351 that first emerged in South Africa) that can infect mice [82, 83]. To circumvent this issue, several approaches have been taken to enable SARS-CoV-2 infection in mice. First, transgenic mice have been made that express human ACE2 [70]. Second, viral vectors have been used to deliver and overexpress human ACE2 in the mouse lung [84]. Lastly, SARS-CoV-2 has been genetically adapted to utilize mouse ACE2 for entry resulting in a virus with high titer replication in the lungs of standard laboratory mice (ie, BALB/c), loss of pulmonary function, severe end-stage lung disease like acute respiratory distress syndrome, and death [85]. In general, any of these mouse models can be useful for antiviral testing. In addition, because the mouse-adapted infection model recapitulates multiple aspects of the human disease, this model may be more useful to study viral pathogenesis and where possible genetically dissect host immune responses driving disease.

Hamster models have been developed to study viral replication, pathogenesis, and transmission [86, 87]. Unlike mice, wild-type SARS-CoV-2 robustly infects the hamster respiratory tract, causing weight loss and lung pathology. The hamster model also recapitulates age-dependent disease severity. The kinetics of viral replication in young hamsters is faster than that in aged hamsters, whereas the immune response in aged animals lasts longer, leading to somewhat more severe disease. Moreover, hamsters can transmit the virus allowing for the study of SARS-CoV-2 transmission. Ferret models have also been established to study viral transmission. Compared with other animal models, the viral replication level and disease severity are milder in ferrets [88].

Several nonhuman primate models of SARS-CoV-2 have been reported, including rhesus macaque, cynomolgus macaque, and

African green monkey [89–91]. In the rhesus macaque model, the infected animals develop mild to moderate clinical signs of disease, develop pulmonary infiltrates on radiographs, and virus shedding in nose and throat swabs is similar to that observed in COVID-19 patients. Although no nonhuman primate models have been established that recapitulate severe COVID-19, these models have been used successfully to show the activity of several direct-acting antivirals and antibody treatments [92–94].

In summary, antiviral models have been developed to support *in vitro* and *in vivo* drug discovery. However, limitations remain, in large part due to the biosafety considerations, to enable more efficient therapeutic development.

Challenges and Opportunities

Data Reproducibility and Assay Comparisons

One challenge to SARS-CoV-2 drug discovery and development is that different research teams have reported different activity, or lack of activity, for the same compound. One side effect of so many research groups converging on SARS-CoV-2 research has been the use of differing models and assays (described above). The adoption of common reference assays, including controls and criteria, would set standards for the field. This would include recommendations on appropriate cell lines and reagents and making sure these are readily available to qualified investigators. For example, HIV repositories exist for small molecules, viruses, and cell lines promoting standardization, which has been beneficial to drug development (although this was implemented over a longer timeline; see for a general example the Biodefense and Emerging Infections Research Resources Repository, BEI Resources). Importantly, the reliance on a single assay model for demonstrating activity is not adequate and orthogonal assays are essential. In addition, testing against standardized positive and negative controls in assays is also important.

Combination Antiviral Therapy

Combination antiviral therapy is expected to be critical to effectively treat infections and minimize the development of resistant strains. Screening approaches can be used to identify synergism of antiviral candidates, and functional/genetic approaches can be used to predict drugged target combinations that should be effective. Moreover, any new therapeutic, especially other RdRp inhibitors, must be tested for antagonism against approved therapies, such as remdesivir, to ensure that unwanted impedance of antiviral activity does not occur in combination. Also, initial clinical studies of potential drug-drug interactions with respect to human pharmacokinetics and tolerability are necessary.

In Vivo Models

Before the SARS-CoV and MERS-CoV outbreaks, very few models of coronavirus pathogenesis existed, but researchers

responding to the SARS-CoV-2 pandemic rapidly took advantage of previous models (eg, human ACE2 transgenic mice). However, the number of researchers able to engage in research with an authentic emerging coronavirus is restricted by the need to perform this work in a BSL-3 laboratory, of which there is a limited number in the United States. Another major gap is that it remains difficult to study long-term effects of either treatment or disease in animal models. From an antiviral candidate perspective, direct-acting compounds against viral proteins can be applied to many animal models, but host-target antiviral candidates (for example, host proteases described earlier) rely on an understanding of the species homolog of the human gene. Current animal models are not adequate for studying extrapulmonary manifestations of disease. Studying comorbidities is challenging in animal models, which is a major weakness given the pathology of COVID-19 in humans, and the anticipation that newly developed antivirals will be effective in reducing mortality in patients with such complicating comorbidities [95].

To date, the FDA has not been requiring animal model data for some clinical trials using agents with known activity during viral infections and well-defined safety based on prior *in-human* use. However, antiviral activity information remains essential in instances when other data, on topics such as the mechanism of action or toxicity, are lacking. Mechanism of action and resistance data is of vital importance. In this context, the designation of a standard accepted *in vivo* model with gold standard positive and negative controls for assessing and comparing antiviral activity of a new therapeutic has not been designated.

LESSONS FROM OTHER VIRUSES AND PREPARATION FOR THE FUTURE

There is a history of antiviral drug discovery and development dating back to the 1960s. While these earliest efforts were largely empiric, the tremendous effort against HIV in the 1980s and 1990s was pivotal in the shifting emphasis to more target-based drug discovery and some of the earliest successes in structure-based and rational drug design. The success of antiretroviral therapy for HIV illustrated the critical importance of combination therapy and adherence in treating chronic infections and highlighted the value of fixed-dose combinations, convenience, and tolerability. These fundamental lessons were subsequently relearned in the discovery and development of direct-acting antiviral drugs for HCV.

HCV drug discovery also emphasized a target-based approach for the development of protease and polymerase inhibitors but, in the end, empiricism was also critical to this amazing success story. The HCV NS5a inhibitors, which became pivotal to most successful combination therapies, were discovered from agnostic cell-based screens that were only made possible by the development of replicon assays. The development of replicons for multiple diverse HCV genotypes was fundamental to

achieving the breadth of activity needed to address both spectrum and resistance, also illustrating the critical value of having the right *in vitro* models to drive the drug discovery process.

Throughout the 1980s, 1990s, and 2000s, advances were also made in therapeutics for HBV, influenza virus, and various herpesviruses. Today, there are more than 90 approved antiviral agents to treat a variety of both acute and chronic viral infections. While there are successful antiviral drugs across multiple distinct target classes, the largest number and broadest class of approved antiviral drugs are those which target viral polymerases. Polymerase inhibitors have been approved to treat infections across both RNA and DNA viruses as well as retroviruses and comprise a myriad of mechanisms, including substrate mimetics such as nucleoside and pyrophosphate analogs but also non-nucleoside or allosteric inhibitors. It is worth noting, even nucleosides can exhibit distinct mechanisms of action, including chain termination, inhibition of translocation, and error catastrophe with unique implications for pharmacology and resistance.

In general, enzyme targets dominate the list of approved antiviral agents, but after polymerase, other enzyme targets are generally unique to different virus families, making it challenging to find drugs that work broadly. The challenge of finding approaches that are effective across a virus or virus family is particularly true for viral entry targets, which can often vary even for a specific virus in cases where multiple receptors or modes of entry are observed. The best clinical illustration of this challenge is the development of CCR5 inhibitors for HIV, where having a diagnostic to differentiate CCR5 versus CXCR4 tropism was critical and became an impediment for the use of such agents in the real world. CCR5 inhibitors are also one of the few examples of a successful antiviral development against a host target, another challenge for antiviral development generally.

Despite a tremendous track record of success, there are lessons from notable disappointments in antiviral drug development. There is always the potential for failure due to toxicity or pharmacokinetics that plague drug development more generally but, in the case of antivirals, poor efficacy can be attributed to an inherent viral diversity or acquired resistance in some instances. One such example is the first-generation HCV protease inhibitors that were obsolete by the time they launched, being replaced by agents with improved resistance profiles and spectrum. While in many cases lessons from the initial clinical disappointments resulted in improved agents, an earlier understanding of genetic diversity and resistance could have avoided many of these early mistakes. Therefore, understanding the impact of genetic diversity and resistance is important even at the earliest stages of target and lead selection.

While it is certainly preferable to develop agents that have a high barrier to the development of resistance, as seen in HIV and HCV, even agents with a low barrier to resistance development

can work when combined if there is adequate pharmacologic coverage. The use of combinations appears to be more important in treating chronic infections, where there is ongoing replication and a larger more diverse population, as compared to prevention where dual and even monotherapy is highly effective. However, it is also relevant in acute respiratory infections like influenza in which resistant variants sometimes emerge during treatment and spread to close contacts. However, even in chronic infection, monotherapy can sometimes work, as is the case in HBV, likely as a consequence of both the biology of the virus and the specific agents.

It is important to note that resistance and antiviral efficacy are inextricably linked to pharmacokinetics. To decrease the probability of resistance development, desirable agent characteristics include high oral bioavailability, high plasma and organ/tissue/site-of-action distribution, long elimination half-life, low intra- and interpatient variability, low probability of drug-drug interactions, and convenient dosing regimens that promote high adherence and are forgiving of missed doses. To maximize the potential for broad impact and global use, oral agents with low cost of goods that are easy to formulate and also suitable for pediatric formulations are critical. For chronic therapy and prevention, drugs that are amenable to long-acting formulations are highly desirable. In addition, understanding where the agent needs to be delivered to treat or prevent infection is key. In respiratory virus infections, intranasal administration is not sufficient to treat or prevent lower respiratory tract disease but may be useful for prophylaxis.

For both acute and chronic infections, timing of initiation of therapy is essential. As has been shown in HIV, early treatment and prophylaxis led to profound patient, public health, and economic benefit. The same has been shown in multiple studies of influenza therapeutics, where the greatest impact of antiviral therapy is observed in the setting of institution of treatment early after disease onset or postexposure prophylaxis. How soon postexposure prophylaxis needs to be administered may vary between different viral infections and antiviral mode of action, but in all preclinical models and clinical studies of respiratory viral infections, sooner is better. In a pandemic setting, this is especially critical. For influenza, insufficient and delayed use of neuraminidase inhibitor treatment resulted in these drugs having a diminished impact on patient mortality during the 2009 pandemic, despite their known effectiveness. The exceptions included countries like Japan that had high levels of antiviral coverage, including children and pregnant women.

A number of potential SARS-CoV-2 inhibitors are being considered for topical administration (eg, intranasal, inhaled) to the respiratory tract. One issue for topically delivered respiratory virus antivirals is whether intranasal administration is sufficient, which in turn depends on the principal site(s) of initial acquisition. Intranasal interferons (IFNs) protect against

rhinovirus and likely common respiratory coronavirus illness. However, neither intranasal IFN nor intranasal zanamivir prevent natural influenza illness. In contrast, inhaled zanamivir is highly effective for prevention of influenza illness (75%–80% in household contacts). It remains to be determined whether antivirals administered intranasally (ie, protecting only the nose) might prevent some SARS-CoV-2 infections. Inhaled investigational agents like IFN- β , which has shown some efficacy in hospitalized COVID-19 patients, and when available inhaled remdesivir, may prove effective in prevention and early treatment.

It is well established that antiviral drugs are highly effective in both treating and preventing infections, if they have the right pharmacologic properties, can be easily accessed, and administered to at-risk populations. Having the appropriate *in vitro* tools and *in vivo* models to develop antivirals are key. Investing in the basic biology to develop these tools, assays, and models, understanding the implications of resistance, and overcoming the barriers that impede rapid access to the use of such drugs once developed are critical for limiting future pandemics and outbreaks. It requires a collaborative effort that crosses public, private, and governmental boundaries.

Preclinical Pharmacokinetics and Formulation

As with any preclinical drug development program, it is essential to understand the physicochemical properties of the drug compound and the resulting pharmacokinetic profile to improve chances of success in demonstrating *in vivo* efficacy. Remdesivir (for example) is given intravenously due to the poor oral bioavailability. Moreover, remdesivir required solubility enhancement using cyclodextrins due to its limited solubility [96]. Preclinical formulation development should take into account drug solubility, stability, and absorption. In the case of poorly absorbed drugs, alternative routes of administration may also be considered. Notably, intranasal and pulmonary administration appears a promising strategy for SARS-CoV-2 antiviral delivery [97].

SUMMARY OF DISCUSSIONS AND PERSPECTIVES ON THE CHALLENGES AHEAD

Overview of the Virus and Therapeutic Approaches

An overarching goal for the coronavirus antiviral program is the discovery of novel compounds that inhibit different steps in the virus replication cycle, and that target diverse coronaviruses both *in vitro* and in animal models. The ability to administer drug by multiple routes (intravenous, intramuscular, subcutaneous, oral, inhalation, and nasal) is highly desirable. A toxicity profile that supports prophylactic (chronic dosing) as well as therapeutic intervention (5 to 10-day dose window) is key. As the current pandemic emerged, the problems associated with clinical development in the absence of a clear understanding of

the virologic and immunologic course of disease became apparent. Important considerations moving forward include determining viral burden, especially in the lower respiratory tract and extrapulmonary sites, establishing the effective therapeutic window, and identifying potent combination therapies designed to improve efficacy and prevent resistance. Even with the availability of effective vaccines, antiviral drugs will be required to protect highly vulnerable populations with diminished immune function or who cannot take vaccines due to allergic reactions.

Targeting Viral Replication Machinery of SARS-CoV-2

Therapeutics against SARS-CoV-2 consist of many subtypes: antibodies, early inhibitors of attachment, spike protein cleavage, fusion inhibitors, RNA polymerase inhibitors, protease inhibitors, and virus release inhibitors, among others. These can be either host or viral targeted therapeutics. One of the most successful methods to mitigate SARS-CoV-2 replication is by direct-acting antivirals; currently, those that target the replication machinery are of high interest. To date, progress has been made in the development of nucleoside analogs, as these are the only class of small-molecule antivirals that have been approved clinically for COVID-19 (eg, remdesivir) [13]. There are advantages and disadvantages of developing nucleoside analogs. Those nucleoside analogs in which the sugar is ribose, called “ribonucleosides,” have had the advantage in that they had been jump started from other viral indications. Advantages include: (1) they mimic natural substances, (2) *in vitro* work has suggested a high threshold of viral resistance, (3) the development pathway is well understood, and (4) tests are in place to detect off-target toxicities. The fact that ribonucleosides tend to be active against other unrelated RNA viruses makes this family of therapeutics desirable for interrogation (both for now and off the shelf for future pandemics) [98–100].

It has proven difficult to establish structure-activity relationships for nucleoside/nucleotide analogs; consequently, nucleoside discovery generally is a trial-and-error process. The current approach is to simply introduce modification on the base or ribosugar, and then test each derivative for activity. In addition, nucleosides require multistep metabolic activation for conversion to the 5′-triphosphate derivative to be inhibitory. This effort can be a challenge as metabolic activation varies between different cell types, which makes selection of assay types and cell types a challenge for downstream evaluation. Furthermore, the delivery of nucleosides to the target tissue of interest can be a challenge. However, there is a wealth of historical data that informs the selection of those nucleosides, which provide a starting place for synthetic efforts. Nevertheless, the chemistry can be challenging to work through the many variations of modifications that are possible on base versus ribosugar. Nucleosides, because they mimic natural factors, also can act through different mechanisms, targeting aspects of the replication machinery of either host

or virus (selectivity). While tools have improved the design of antiviral nucleosides, these compounds must be tested in whole-cell screens and must be evaluated for the potential off-target effects (mitochondrial or host cell compartment interactions). Lastly, the design of alternative delivery options, for example, oral delivery, can be challenging. Tactics to make all the information surrounding the work in nucleoside therapeutics more widely known across disciplines, including the assays to determine antiviral effect, must be made more generally available.

Proteases (Viral and Hosts)

An obvious target is viral and host proteases, as these proteins are important for viral entry and processing. Of benefit is that libraries of HIV and HCV protease inhibitors provide a logical starting point for synthesis. Repurposing protease inhibitors provides an invaluable and rich resource, especially for combination therapies. Proteases have been demonstrated to be broadly druggable, and new preclinical candidates will emerge. Currently, success has been extremely limited in contrast to that achieved with HIV and HCV infections.

Emerging Targets and Emerging Modalities

Knowledge of the replication cycle of SARS-CoV-2 indicates that there are numerous other targets of the viral or host genome or proteome that are largely unexplored and could be suitable for drug discovery. The fact that these targets are unexplored makes this route of therapeutic discovery and development more contingent on unknown factors. These unknown risks and benefits must be recognized, but it should not be a factor that impedes progress.

Additionally, many new modalities have arisen to target both conventional and novel viral and host targets. These new modalities offer advantages for speed of discovery and development, potency, selectivity, and cost of goods. Continued exploration of these emerging targets and modalities should be pursued in parallel with traditional direct-acting antivirals.

Preclinical Tools

When choosing the preclinical tools to test compounds *in vitro* and *in vivo*, the choice of cell type is crucial for cell assays. Orthogonal assays as well as AI are necessary tools to complement discovery efforts [101]. *In vivo* models can help determine if a drug is capable of engaging a target tissue. In particular, animal models provide a critical tool for the assessment of drug distribution, including to the relevant site(s) of viral replication. Furthermore, the half-life, oral bioavailability, metabolism, distribution, microsomal stability, etc. can all be evaluated in animal systems [102]. However, animal models may fail to determine if metabolic differences can drastically alter the way a drug is metabolized. Furthermore, it should be emphasized that a drop in viral burden is not a validated end point for efficacy. Each animal model has its own set of peculiarities

and other models could be considered. Especially for SARS-CoV-2 (and other respiratory viruses), the air-liquid interface human airway cell model may provide insight into tissue site needs [103]. Ultimately, many therapeutic candidates succeed in preclinical development and fail in human clinical trials, and building a translational science pathway for antiviral preclinical development has significant unmet needs.

Clinical Trials and Lessons From Other Viruses and Preparation for the Future

When considering lessons from previous antiviral drug development, clinical trial design and execution should be carefully reviewed. Decrease in viral burden is not a traditionally validated end point, with the exception of HIV, HCV, and cytomegalovirus after hematopoietic stem cell transplantation. However, for SARS-CoV-2 antiviral candidates, the lack of antiviral efficacy in early clinical studies of prevention or treatment of mild illnesses should raise concerns about advancing such candidates further. For agents demonstrating antiviral activity, what should antiviral clinical trial end points be? For SARS-CoV-2, possibilities may be dependent on the indication (eg, preexposure or postexposure prophylaxis, treatment) and target population (eg, early treatment in outpatients to ameliorate illness, prevent disease progression, and/or reduce transmission; treatment in seriously or critically ill hospitalized patients), but these are by no means comprehensive or definitive. The logistical issues are many, such as defining diverse patient populations or, often forgotten, the staffing and supply needs to accommodate the appropriate trial. Standardization of platforms for each stage of development, including the clinical trials, would be a helpful step for all. This has been achieved in the pandemic response in part through creation of large pragmatic, adaptive trials in hospitalized COVID-19 patients like RECOVERY in the United Kingdom, WHO's Solidarity Trial, and ACTT and ACTIV in the United States. These platforms have provided key outcomes data on therapeutics that helped (ie, dexamethasone, baricitinib), those that did not (hydroxychloroquine, lopinavir-ritonavir, azithromycin), and those with inconsistent results (eg, remdesivir). Availability of databases and algorithms for AI to provide computational oversight for *in vitro* and *in vivo* models and clinical trial design as well as openly sharing negative data among all research groups is useful to avoid rabbit holes that are a waste of time and money. Standardized end points would also enable "basket antiviral trials" that test multiple antivirals (or combinations) with a single placebo arm.

Ultimately, for the development of any drug, the most important factor is to guarantee that the lead is safe for human administration [104]. As emphasized, the early stages of the drug discovery and development process should include considerations regarding what the final product insert will recommend as the indication. Considerations would include: will a

compound be used for prophylaxis (which requires a higher safety threshold), and what formulation options are required according to the route of delivery? Drug delivery to the site of viral replication/disease must be included in the development pathway and crucial treatment paradigm. Establishing these goals at the outset ensures the appropriate design, particularly outpatient or inpatient, with or without multiorgan involvement. In the medicinal chemistry field these are referred to as “target product profiles.” The breadth of knowledge critical for drug discovery and development is vast. A translational science approach with a combination of skill sets from virologic and immunologic backgrounds as well as chemistry, toxicology, and other fields are important factors of success.

Forming Partnerships

Forming product development partnerships (PDPs) around direct-acting antivirals is a powerful means of rapidly discovering and developing antiviral therapeutics. PDPs are synergistic initiatives between academic innovators at the cutting edge of their discipline and biotechnology/pharma-based drug developers, who can rapidly move clinical development candidates forward through preclinical and into clinical development [105]. Formation of these partnerships is absolutely necessary to maximize the possibility of success in a time and cost-efficient manner. Working with regulators during a pandemic is essential. Defining the resources that are needed and are already available is key. Both NCATS and NIAID have preclinical development resources that are readily accessible.

Notes

Disclaimer. The views expressed by J. O. R. are his and do not necessarily represent the position of, nor imply endorsement from, the US Food and Drug Administration.

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