Sprint discovery of direct-acting small molecule antivirals: Learnings from COVID-19

Annette von Delft[1,2,3,*], Matthew D. Hall[4], Ann Kwong[5], Lisa A. Purcell[6], Kumar Singh Saikatendu[7], Uli Schmitz[8], John A. Tallarico[9], Alpha A. Lee[3,10,11,*]

1. Centre for Medicines Discovery, Nuffield Department of Medicine, University of Oxford, Oxford, UK
2. Oxford Biomedical Research Centre, National Institute for Health Research, University of Oxford, Oxford, UK
3. The COVID Moonshot Consortium
4. National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Rockville, Maryland 20850, USA.
5. Pardes Biosciences, 2173 Salk Ave Suite 250, PMB 052 Carlsbad, CA 92008, USA
6. Vir Biotechnology, San Francisco, CA 94158, USA
7. Drug Discovery Sciences, Takeda California, Inc., 9625 Towne Center Drive, San Diego, CA 92121, USA
8. Gilead Sciences, Inc, Foster City, California, USA
9. Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA
10. PostEra Inc, 1 Broadway, MA 02142, USA
11. Cavendish Laboratory, University of Cambridge, Cambridge CB3 0HE, UK

Abstract

The COVID-19 pandemic is a stark reminder that a broad pipeline of antivirals against viruses of pandemic concern is an essential component of pandemic preparedness. During the COVID-19 pandemic, a wave of rapid and collaborative drug discovery efforts took place in academia and industry, culminating in several therapeutics discovered, approved and deployed during a two-year time horizon. This article summarises the collective experience from multiple pharmaceutical companies active in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antiviral discovery, surveying key stages in the drug discovery process: target selection, medicinal chemistry, antiviral assays, animal efficacy, and attempts to pre-empt resistance. We outline current antiviral drug discovery and development opinions and learnings, and propose strategies that could accelerate future efforts. We argue that a key bottleneck is the lack of quality chemical probes that can build conviction around understudied viral targets and serve as a starting point for drug discovery. Considering the small size of the viral proteome, comprehensively building an arsenal of probes for proteins in viruses of pandemic concern is a worthwhile and tractable challenge for the community.

*These authors contributed equally. For correspondence: Annette.vondelft@cmd.ox.ac.uk (Annette von Delft) and alpha.lee@postera.ai (Alpha A. Lee)

Introduction

Viral outbreaks are one of the gravest public health risks of our times. The ongoing Coronavirus Disease 2019 (COVID-19) pandemic has claimed over 6 million lives, and several broader global trends make pandemics more likely in the future. Climate change, as well as the impact of people moving into and destroying wildlife, increase human-animal interactions and the risk of zoonotic spillover [1,2]. Warming surface temperature also increases the geographic extent that is hospitable to viral vectors such as mosquitos and ticks, potentially increasing the spread of arboviruses [3]. Globalisation and prevalence of global travel can rapidly turn a local epidemic into a global pandemic [4]. As such, developing effective therapeutics against current and future pandemics should be a global public health priority.

Prior to the COVID-19 pandemic, the focus of antiviral development has been on Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV), accounting for more than 67% of approved antivirals [5]. The routine drug discovery and development timescale can be of the order of decades, especially for first-generation therapeutics against a virus. COVID-19 combined the attributes of an acute, severe and rapidly transmissible virus. For the first time, the translational science sector has successfully executed rapid drug discovery campaigns and developed novel antivirals amid a fast-moving pandemic. Within two years there were two oral therapeutics with Emergency Use Authorizations (EUA): Paxlovid (Pfizer) and Molnupiravir (Merck; developed originally for VEEV), and several clinical stage investigational oral therapeutics such as S-217622 (Shionogi), PBI-0451 (Pardes), bemnifosbuvir (AT-527) (ATEA) and EDP-235 (Enanta). In addition, Remdesivir (Gilead; developed originally for Ebola), an IV small molecule therapeutics, was approved early on in the pandemic.

This perspective draws from a roundtable discussion between biopharma companies and public sector organisations with substantial research and development efforts in COVID-19, collectively leading to 2 approved therapeutics (Remdesivir, Soltrovimab), one Phase 2 (PBI-0451), and one Phase 1 (GS-5205) asset at the time of writing. We will outline key learnings from the antiviral discovery sprint and articulate remaining open questions, specifically focusing on target selection, resistance, antiviral assays, in vivo models and medicinal chemistry strategies.

Target selection and validation

Antiviral therapeutics can be segmented into host-directed and direct-acting strategies. Host-directed antivirals target human proteins that are essential in the viral lifecycle. Significant effort has been expended in finding host-directed therapeutics against COVID-19 [6,7], most notably through numerous repurposing screens. Some of these went into clinical trials, through platform trials such as Accelerating COVID-19 Therapeutic Interventions and Vaccines and Randomised Evaluation of COVID-19 Therapies trial (ACTIV, RECOVERY)

[8,9] as well as company-sponsored trials. Nonetheless, to date there has been no approved host-directed antiviral therapeutic against COVID-19. It is argued that the advantage of a host-directed approach is a potentially higher barrier to antiviral resistance, as well as broad spectrum activity if the target is employed by multiple viruses [10]. Nonetheless, downsides include possible host pathway-mediated (on-target) toxicity, lower efficacy compared to direct-acting antivirals as the viral life cycle may leverage multiple redundant targets, and poor translation of in vivo models. Historically, the only successful host-directed antivirals were interferon for HCV and HBV, and CCR5 antagonists for HIV [11], as well as cyclophilin inhibitors such as Alisporivir in late stage clinical development for HCV [12]. For these reasons, most approved antivirals directly target viral proteins, and the focus of this article will be targets associated with SARS-CoV-2.

Prior to embarking on a drug discovery effort, target selection is crucial: The ideal antiviral target is essential for the viral life cycle (e.g. has a tractable mechanism of action), can be inhibited by small molecules with drug-like pharmaceutic properties, and has a high fitness barrier to mutation [13]. We identify several key concepts that aid SARS-CoV-2 antiviral target selection (**Table 1**).

Validated antiviral mechanism of action. Antiviral targets with previous clinical evidence, showing that target inhibition leads to therapeutic antiviral effects, have a lower translational risk ("best-in-class" approach). This is a high bar to meet, and these targets may not be rapidly available in an emerging pandemic setting. However, evidence from other viruses may provide confidence in the target, when certain viral replication mechanisms are shared and can help to demonstrate that the target is salient. We can establish "target-class confidence" if there are multiple approved therapeutics against the same target class in multiple viruses. We note that ongoing efforts by the National Institute for Allergy and Infectious Diseases aims to define and develop therapeutics "prototype pathogens" and "priority pathogens", key viruses in viral families of pandemic concern [15].

In the absence of clinical validation, a target is more credible if there is understanding of the protein function, and biological evidence demonstrating that ablating protein function directly impacts viral replication in vitro or in vivo. This can be achieved via chemical probes (discussed below), or a reverse genetics approach of introducing mutations in the protein of interest.

The indirect pathway of inhibiting viral proteins which are responsible for evading host immune response is a less trodden path, as there might be multiple viral mechanisms to suppress host immune response. Likewise, care must be taken in targeting steps in the life cycle such as viral entry, where multiple pathways for infections and cell to cell spread have been shown to exist for some viruses.

Chemical probe validation. The goal of an antiviral therapy is the chemical inhibition of the target. As such, key questions are: (1) whether there are sites on the protein that can be engaged by a small molecule ("druggable"), (2) whether engaging these sites modulates protein function, and (3) whether a chemical probe demonstrates that modulation of protein function translates to viral inhibition in cellular assays. These are conceptually separated but interdependent questions, in order of saliency to target validation.

The existence of complete chemical probes for validation - small molecules that potently inhibit the target with corresponding cellular antiviral activity, with changes in the potency of inhibition translating to changes in antiviral activity, so-called Structure-Activity Relationship (SAR) - helps build confidence that a target is validated and tractable. The availability of meaningful functional assays and linked structural data may greatly facilitate the development of chemical probes for previously untargeted viral proteins. Whilst many chemical probes have been developed for human targets, less chemical probes and structural data are available for viral targets.

Sequence conservation. In addition to direct clinical evidence or strong mechanistic evidence, a way to evaluate the saliency of a target is via evaluating sequence conservation through alignment, both across the virus family, or within circulating variants. There are two reasons why conserved targets are perceived as more robust: (i) if a target is essential to the viral life cycle it will accumulate fewer mutations, since mutations may impact viral replication and fitness; (ii) even if a mutationally flexible target is essential, it is harder to drug because the molecule will need to potently inhibit all replication-competent quasispecies; and (iii) targeting a conserved viral target increases the likelihood of developing a broad-spectrum antiviral, an important attribute when developing drugs for pandemic preparedness. Sequence and structural conservation can be evaluated across the entire protein, or within the active binding site.

Clinically validated targets

To date, the only oral SARS-CoV-2 therapeutics in clinical use or late-stage clinical trials target either the main protease (nsp5-Mpro) or the RNA dependent RNA polymerase (RdRp) (**Figure 1**). Surveying the clinical and preclinical antiviral pipeline in the public domain, we anticipate RdRp and Mpro-directed therapeutics to remain the most prevalent targeted proteins for the next 3-5 years.

nsp5-Mpro

The SARS-CoV-2 genome encodes 2 polyproteins and 4 structural proteins. The polyproteins are cleaved by the cysteine proteases nsp5-Mpro (responsible for cleavage at 11 positions) and nsp3-PLpro (3 cleavage positions, discussed below) to liberate shorter viral proteins crucial for viral replication (such as the RdRp, discussed below) and evading the host immune response. For example, Mpro has been observed to directly cleave NLRP12 and TAB1, two modulators of inflammatory pathways that might point to a molecular mechanism for enhanced production of cytokines and the resultant inflammatory response observed in COVID-19 patients [17].

Numerous companies initiated internal programs targeting Mpro early in the pandemic, and several Mpro inhibitors are now under EUA or in the clinical pipeline (**Table 2**). Mpro is an attractive target based on the rare confluence of: (1) Mechanistic understanding - protease function for polyprotein processing is well-characterised, assayable, and inhibition of Mpro directly suppresses viral replication, as observed in many viruses, such as the closely related SARS-CoV, as well as HCV, HIV, and Human Rhinovirus (HRV); (2) NSP5-Mpro is a cysteine protease, a well-characterized class of enzymes known to be druggable and is distinct in amino-acid sequence and cleavage specificity relative to known human cysteine

proteases; (3) Clinical precedent - multiple HIV and HCV protease inhibitors are in clinical use, and a HRV protease inhibitor reduced the proportion of subjects with positive viral cultures in a Phase 2 clinical trial [18], leading to confidence in inhibition of viral proteases as a target-class; and (4) Chemical probe validation - multiple SARS-CoV chemical probes targeting Mpro were reported after the 2003 SARS-CoV epidemic in Asia [19,20]. Later efforts based on early work against the Norwalk virus 3C protease lead to the development of GC376, a compound that demonstrates good activity across Norovirus, Picornaviruses and Coronavirus, as well as a reversal of lethal disease in a cat coronavirus model, feline infectious peritonitis (FIP) [21,22]. As SARS-CoV and SARS-CoV-2 Mpro share 96% sequence similarity, some chemical probes were rapidly redeployed to SARS-CoV-2. In fact, PF-00835231 was selected as a development candidate in response to the 2003 SARS-CoV pandemic, with potent cellular antiviral activity and favourable preclinical DMPK properties, but discontinued before entering into clinical development as the epidemic had been contained and there was no active patient population [23,24]. Further, the rapid availability of the SARS-CoV-2 MPro structures has facilitated structure-based design efforts **(Figure 2)** [25,26].

nsp12-RdRp

The replication of RNA viruses requires a mechanism to synthesise viral RNAs. The RNA-dependent polymerase (RdRp) catalyses the replication of RNA from a RNA template, synthesising a RNA strand that is complementary to the template. Inhibiting the RdRp therefore inhibits viral replication. Similar to Mpro, RdRp as a target shows the confluence of: (1) Mechanistic understanding - the structure and function of RdRp are well-understood across RNA viruses with an essential role in viral replication. Additionally, RdRps are not encoded by human cells, though inhibition of human RNA polymerases is a source of off-target toxicity for RdRp inhibitors [27]. (2) Clinical precedent - multiple RdRp inhibitors are in clinical use or development for HIV, HCV, RSV, influenza A, and influenza B [28], increasing confidence in the coronavirus RdRp as a relevant target. (3) Chemical probe validation - a wide range of RdRp inhibitors have been developed. Some have been successfully deployed against SARS-CoV-2 infection, such as remdesivir [29], and subsequently molnupiravir [30]. In particular, remdesivir's broad-spectrum antiviral activity against coronaviruses was published prior to the SARS-CoV-2 pandemic [31,32], as well as its biochemical mechanism of action [33]. Although engaging the same target, the mechanisms of remdesivir and molnupiravir are different: initially, both molecules require metabolic activation by the endogenous machinery of the cell and once activated, remdesivir leads to delayed termination of RNA replication [34], whereas molnupiravir leads to mutated RNA products [35]. Notably, other repositioned RdRp inhibitors such as favipiravir or sofosbuvir, albeit showing some antiviral activity in selected cellular assays, were not shown to impact mortality and hospital admissions in SARS-CoV-2 clinical trials [36,37]. (4) Sequence conservation - the catalytic site of RdRp is broadly conserved across coronaviruses and variants of SARS-CoV-2 (c.f. **Table 1**) [14].

Targets with chemical probe validation

nsp3-PLpro

PLpro is also a cysteine protease with a papain-like fold, one of the two proteases in SARS-CoV-2, responsible for processing three cleavage sites in the N-terminal part of the polyproteins to produce mature nsp1, nsp2, and nsp3 (**Figure 1**). The active site contains a classic catalytic triad, composed of Cys112–His273–Asp287. Apart from its essential role in viral replication, PLpro cleaves ubiquitin, ISG15 and IRF3, known regulators of host innate immune pathways [17,44]. Non-covalent inhibitors have been found against SARS-CoV-1 [45] and SARS-CoV-2 PLpro [46–49]. In both cases, PLpro inhibition correlates with antiviral activity.

Targets with genetic evidence

Viral replication

nsp13-helicase

Nps13 is part of the RdRp replication complex and catalyses the unwinding of RNA in a 5′ to 3′ direction [50,51], as well as relevance for the proofreading and template switching functions of the replication complex [52,53]. There is no reported chemical probe against coronavirus helicase, although a crystallographic fragment screen suggests that it is a tractable target [54]. However, viral helicases have been pursued for other infections: Amenamevir for the treatment of reactivation of varicella zoster virus (shingles) is approved in Japan [55], and BAY 57-1293 (Pritelivir) is currently in Phase 3 clinical trial for herpes simplex virus [56–58].

Evading host immunity

nsp3-mac1

As part of the innate immune response, host ADP-ribosyltransferases transfer ADP-ribose onto viral proteins, ultimately contributing to the suppression of viral replication. The viral macrodomain nps3-mac1 counteracts this innate immune response by cleaving ADP-ribose already transferred onto viral proteins. Viral macrodomains are found in corona, alpha, rubi, and herpes viruses [59,60], and it has been shown that macrodomain mutations disrupt catalytic activity and decrease virulence [61]. There is no reported chemical probe against Mac1, nor clinical evidence for inhibiting viral macrodomains, although a crystallographic fragment screen has been done revealing starting points for small molecule inhibitor synthesis and suggesting that it is tractable for small molecule development [59].

nsp14 and nsp16 methyltransferases

Methyltransferases catalyse the transfer of a methyl group from *S*-adenosyl methionine to RNA substrates. In complex with nsp10, nsp14 catalyses the N-methylation of guanosine, whilst nsp16 completes the formation of the RNA cap by 2'-O-methylation of ribosyl-adenine [62]. The formation of the RNA cap subverts the host's innate immune responses [63]. Recent structural biology work elucidating the mechanism of methyl transfer [64,65], and chemical probes targeting the SARS-CoV-2 nsp14 methyltransferase have been reported [66,67]. However, to date there is no evidence that chemical inhibition translates to cellular

antiviral activity, nor clinical precedent for inhibition of viral methyltransferases. We note that nsp14 also encodes an exoribonuclease activity that performs a proofreading function and antagonises the innate immune response [68].

nsp15-endonuclease

Nsp15-endonuclease is a RNA uridylate-specific endoribonuclease that is part of the EndoU family [69]. Members of this family of enzymes act on RNA, cleaving 3' of uridylate and thereby generating a 2', 3' cyclic phosphate and 5'-hydroxyl termini [70]. Nsp15 acts by cleaving viral RNA that would activate the hosts' innate immune response [71–73]. There is no approved therapeutic against coronavirus endonucleases, nor chemical probes, although a crystallographic fragment screen has been performed suggesting that starting points for small molecule inhibitor synthesis exist [74]. The endonuclease has been previously targeted in influenza, where an inhibitor of the cap-dependent endonuclease, Baloxavir marboxil (Xofluza) has been in clinical use for several years [75]. However, resistant viruses emerged in baloxavir-treated subjects at a frequency ranging from 3-11% in adults to >23% in children [75,76]. Further, viral nsp15 domains are highly conserved amongst all nidoviruses [77].

Medicinal chemistry

Once target selection has been made, the medicinal chemistry campaign is an iterative process of designing, making and testing molecules to progress chemical starting points to development candidates. Setting a realistic goal for the profile of a therapeutic is important in terms of designing an appropriate medicinal chemistry strategy, so that the final product has sufficient therapeutic value whilst ensuring efforts are not wasted into over-optimization. These goals are typically segments into clinical (Target Product Profile; TPP), or molecular (Target Candidate Profile; TCP) [78]. The TPP describes clinical attributes of a therapeutic, whereas the TCP describes the molecular attributes (e.g. target engagement, cellular antiviral response, safety pharmacology) that the molecule must fulfil. Here, we discuss the TPP as it informs the development of TCPs, which are target-specific.

Target Product Profile

In an ideal antiviral drug discovery setting, the target product profile (TPP) for a directly acting antiviral aims for an orally available drug that is administered once daily or, for acute infection like SARS-CoV-2, even once only (**Table 3**). In addition, a wide treatment window is desirable, in order to be relevant to patient populations with limited access to rapid diagnostics.

Yet for an immediate pandemic response, many of these specifications are a luxury, and less stringent TPP requirements may be tolerated (**Table 3**). Considering the urgency of the situation and starting with an analysis of the most vulnerable population, the following limitations alone or in combination may be acceptable for first generation antiviral therapies: (i) Suboptimal dosing regimens up to three or four times a day that may impact patient compliance; (ii) Suboptimal application routes including intravenous formulations for selected high-risk patients. Albeit likely not practical for wide-spread treatment of early infection, the highly efficacious intravenous compound remdesivir received EUA and then full FDA approval during this SARS-CoV-2 pandemic [79], and the first SARS-CoV-2 main protease

inhibitor entered clinical trials as an intravenous formulation [80]; (iii) A distribution, metabolism, and pharmacokinetic (DMPK) profile of free drug concentration Cmin>EC90 for 90% of patient population, may be acceptable for a first-generation antiviral during an evolving pandemic (**Figure 3**). This is generally considered to be the minimum coverage to achieve efficacy, but higher coverage may be desirable for second-generation antivirals with better understanding of the emergence of resistance; (iv) Consideration of narrower patient cohorts and a willingness to monitor potential drug-drug interactions (DDI). Clinically manageable drug-drug interactions, co-dosing with pharmacokinetic enhancers, or mechanisms of action precluding the use in selected patient cohorts, e.g. women of childbearing potential, may be acceptable in a pandemic for selected high-risk patient cohorts. (v) Acceptance of short therapeutic windows. For some viral therapeutics, such as Oseltamivir for influenza, rapid treatment is required for antiviral efficacy [81]. However, clinically it can be challenging to prescribe and distribute a drug to a patient within 48 hours of symptom onset. Albeit not ideal, short windows may be acceptable in a pandemic setting and even beyond. However, considering the significant logistical challenge associated, it is preferable if antiviral therapy remains efficient if treatment start is delayed for up to 5 days after symptom onset.

For SARS-CoV-2, there are several clinical observations that may require additional potential amendments to the TPP. First, bi-phasic viral kinetics, or “rebounds”, have been observed in treated and untreated patients [82,83]. Second, a series of chronic neurological, cardiovascular and gastrointestinal symptoms, known Post-Acute COVID-19 Syndrome (PACS) or colloquially “long COVID” are observed in some patients [84,85].

The etiology of these observations are yet unknown. However, there are several factors that may be at play, and may have implications on the TPP. First, the free concentration of the drug may be insufficient to adequately suppress viral replication, thus targeting a Cmin covering multiples of the EC90 in the TPP might be required. Second, the viral kinetics might require longer treatment durations. Third, untargeted viral reservoirs might persist. In particular, the impact of SARS-CoV-2 infection on the central and peripheral nervous system in the acute and chronic phase remains unclear [86,87]. This may suggest that a different tissue distribution profile is desired in the TCP. Finally, for PACS, immune triggers might be of clinical relevance, potentially requiring a host-directed approach beyond direct acting antivirals.

Finally, the TPP might be refined to accommodate features relevant to compounds for pandemic preparedness and increasing the barrier to resistance. Compounds with an increased antiviral treatment spectrum, aiming to cover several viral strains from a viral family - or even across viral families - might be desirable. However, increasing spectrum comes with medicinal chemistry challenges: a compound needs to achieve the (often conflicting) goals of simultaneously inhibiting proteins from related viruses, yet avoid related host proteins and causing potential off-target effects.

Drivers of medicinal chemistry acceleration

The main drivers of an accelerated drug discovery effort during a pandemic are the upfront availability of high-quality chemical matter, the willingness to move at risk, a reconsideration of what are the essential attributes versus the ideal therapeutic profile is required to address

the immediate unmet medical need, and the funding to do the work. More broadly, a rapid campaign requires a sense of organisational commitment and alignment with management to release significant financial investment at risk to execute fast, safe and regulatory rigorous campaigns.

Availability of high-quality chemical matter

Sir James Black, winner of the 1988 Nobel Prize in Physiology and Medicine, famously stated that “the most fruitful basis for the discovery of a new drug is to start with an old drug” [88]. One of the most pressing problems with antiviral drug discovery against a novel target is the availability of high-quality chemical matter. The only two approved RdRp-targeting antivirals, remdesivir and molnupiravir, were developed before the pandemic. Exceptionally rapid drug discovery efforts were executed against the Mpro: One example is the effort based on a peptidomimetic scaffold optimised for SARS-CoV in 2003, with low oral bioavailability only supporting IV dosing. The scaffold in turn shares structural similarity with rupintrivir, a HRV antiviral developed in the 1990s [89,90]. A significant medicinal chemistry campaign was required to optimise oral bioavailability, leading to the novel SARS-CoV-2 MPro inhibitor nirmatrelvir [38,39]. Another example is S-217622, a clinical candidate developed by Shionogi, which is structurally differentiated from reported protease inhibitors [25], though related to a P2X3 antagonist in the Shionogi clinical pipeline [91]. These pockets of exceptional drug discovery appear to validate Sir James’ adage, but also reflect the reality of pre-clinical drug development timelines. By leveraging pre-existing validated chemical matter, the timeline to first-in-human studies is dramatically shortened.

Looking ahead, for pandemic preparedness, we argue that a campaign to develop quality chemical probes and leads against targets in the viral proteome is a key opportunity for investment, to get out in front of and ahead of the next pandemic. Similar to efforts in systematically finding probes against human targets [92], efforts towards finding chemical probes against every viral protein for virus families most likely to produce the next pandemic infection is a worthwhile endeavour for the scientific community to pursue. The viral proteome (29 proteins encoded in the SARS-CoV-2 genome) is orders of magnitude smaller than the human proteome. Thus the level of investment required to execute such an effort is likely to be much less than the human and financial toll of a future pandemic.

Hit-finding technologies

A prerequisite of running a medicinal chemistry campaign is quality biochemical assays. This is often a chicken-and-egg problem with quality chemical matter - chemical probes help validate an assay by generating the confluence of biophysical, biochemical, structural biology and antiviral efficacy data. Establishing a suite of high throughput orthogonal biochemical and cell-based assays around a target is one of the challenges of rapid drug discovery against novel viral targets. As viral targets are typically conserved, preemptively developing and open-sourcing assays, making these assays available to the field for key viral proteins, is a pre-competitive activity that should aid future drug discovery and pandemic preparedness efforts.

Several enabling technologies have been deployed successfully during the pandemic to accelerate the process of hit finding against numerous targets. Experimentally,

crystallographic fragment screens have seen prolific successes against multiple targets. The confluence of high throughput crystallography, automated processing pipeline, and expanded fragment libraries appears to have paid off and consistently delivered dense fragment hits against Mpro [93], nsp3-Mac1 [59], helicase [54] and endonuclease [74]. The latter 3 targets are novel targets with no pre-existing hits. However, going from fragment to lead remains a challenge. Creative approaches such as The COVID Moonshot, which used crowdsourcing to generate ideas at the fragment-to-lead stage via fragment merging [94], have been attempted during the pandemic. The resulting lead is under preclinical development though still some distance away from human clinical evaluation [26]. Organisationally, several pharma companies in this roundtable have established shared hit-finding efforts, where blinded libraries of compounds were screened for Mpro biochemical activity.

Computationally, structure-based virtual screening yielded multiple successes. The discovery of S-217622 employed structure-based virtual screening and followed by pharmacophore filtering to generate novel non-covalent hits against Mpro [25]. An unrelated effort similarly employed virtual screening to discover novel chemotypes that led to potent inhibitors with broad-spectrum antiviral activity [95]. Beyond hit finding, a recent publication has employed Free Energy Perturbation (FEP)-guided optimisation to morph a hit from a repurposing screen, Perampanel, to a potent inhibitor [96]. Note that reported SARS-CoV-2 successes to date in computational chemistry are all targeted towards Mpro, a target with well-developed chemical matter that predates SARS-CoV-2; internal computational hit finding campaigns against novel viral targets performed by members of this Roundtable were much less successful.

Beyond lead-optimisation: Time, Risk and Cost in process development

Beyond the discovery stage, developing and executing process-scale chemistry for late-stage drug development, clinical trials and eventual market distribution requires significant resources. To execute a drug discovery sprint, a holistic view on chemistry timelines needs to be considered. Process chemistry should be involved as soon as lead scaffolds emerge. This allows process development towards key building blocks or intermediates to commence before candidate declaration, thus reducing the lead time. Concomitantly, the commercial availability of building blocks, and the scalability of synthetic routes, should be factored into the medicinal chemistry campaign.

Further, we argue that a critical juncture for a sprint discovery campaign is when to commit to scale up, and to what scale/quality. Conventional drug discovery usually takes a stage gate approach, with separate scale up campaigns for Dose Range Finding (DRF) and GLP toxicology, and manufacturing for clinical evaluation. This mitigates risk as the compound can fail at each stage of the development process. The most successful sprints during the pandemic benefited from providing a large upfront investment to trigger all chemical manufacturing stages in parallel, when the evidence for the candidate is still at the level of cellular antiviral assay and single species PK. This has further upstream impact, in terms of the need to reserve process chemistry resources and pilot plants at an early stage. This incurs an opportunity cost as delays in the drug discovery campaign, or negative readout

during the development process, can mean these expensive downstream resources become underutilised.

Ultimately, speed comes at a cost, which manifests both in actual cash expenditure and increase in risk. If the aim is to impact an ongoing pandemic via a drug discovery sprint, we argue that the investment has to be end-to-end, and stakeholders need to have a “go hard or go home” attitude. Piecemeal investments can end up setting up significant roadblocks down the road, and acceleration in one phase (e.g. discovery) becomes throttled by delays later on. This point is perhaps particularly poignant for public sector investments and grants into drug discovery, as outsized risk cannot be directly economically compensated. One needs to be mindful that stage gates and milestones can lead to significant delays by preventing the parallelization of time-consuming activities.

In vitro cell culture models for SARS-CoV-2

Cellular antiviral infection models are paramount to the identification of effective small molecule inhibitors. A critical part of the medicinal chemistry effort is to understand the variation in biological activity through a cellular assay as a function of chemical structures, which is only feasible with a low variance system. Especially in the later phases of drug discovery campaigns, cellular assay throughput is crucial to driving the campaign and ideally matches chemical synthesis. As assay data is often used to strategically rank order compounds, quantitatively comparing potencies of different compounds *across* assays should be cautioned.

In antiviral drug discovery, many assays use pathogenic infectious viral strains and therefore have to be run in laboratories with higher containment capabilities (i.e. Biosafety Level (BSL) 3, select agent or BSL4), complicating assay logistics and overall accessibility of relevant antiviral assays and ultimately impacting on assay throughput. With these restrictions in mind, the roundtable agrees that for driving drug discovery, reproducible antiviral cellular assays that can be run in a high-throughput setup are much preferred over noisy and/or low throughput assays, regardless of purported biological relevance. A critical component of the assay is tracking variance, robustness and reproducibility, with statistical measures such as Z scores.

In the roundtable’s internal campaigns, cellular assays are generally grouped into high-throughput Tier 1 assays, and lower throughput Tier 2 assays (**Figure 4**). Tier 1 cellular assays are reliable and scalable 2D cell cultures infected with a SARS-CoV-2 strain that are easy to run. With these, efficacy of antiviral inhibitors is generally assessed by adding different concentrations of compound to the culture (either prior or after infection), and viral replication is subsequently measured using a variety of methods. In contrast, “Tier 2 cellular assays” are often lower throughput and may utilise primary cells with a higher disease relevance.

A wide range of experimental parameters influence the use and scalability of cellular assays (**Textbox 1**), including the type of cell lines chosen, the infecting viral strain, and the experimental readout used. Modifications in these parameters can impact reported efficacy measurements, and may contribute to the significant lab-to-lab variability reported in antiviral

efficacy measurements. Since drug discovery efforts rely on comparability of results over time, early assay optimization and consistent use of a fixed protocol within an established facility is essential to medicinal chemistry efforts and understanding of structure-activity relationships.

Cell lines

Commonly used Tier 1 cell models.

For the assessment of antiviral activity against SARS-CoV-2, several cell lines are routinely used. Initially, many assays focused on the African green monkey cell line VeroE6, an animal cell line commonly used for antiviral assays and previously used for SARS-CoV replication [97]. VeroE6 cells are very susceptible to SARS-CoV-2 viral infection and grow easily. However, as VeroE6 cells are not a human cell culture line, nucleos(t)ide analogs (NUCs) often show decreased activity in these cells due to inefficient metabolic activation [98]. In addition, SARS-CoV-2 mutates rapidly in VeroE6 cell lines [99,100], commonly accumulating changes in the furin cleavage site of the spike protein, amongst others [99,101,102]. Further, the high expression of functionally active P-glycoprotein (p-gp) efflux pumps may require the additional use of p-gp inhibitors to assess antiviral activity [38].

The non-small-cell lung cancer cell line Calu-3 is another commonly used line that supports SARS-CoV-2 replication [103], albeit at significantly lower levels than VeroE6 cells [98]. Further, low growth rates and irregular growth patterns of Calu-3 cells have led to difficulties to scale up and automate high-throughput assays with this cell line. Other human cell lines in which SARS-CoV-2 replicates efficiently include the intestinal cell line Caco-2, in line with clinical manifestation of SARS-CoV-2 symptoms, and the liver cell line Huh-7 [104,105].

Overexpression of entry receptors.

SARS-CoV-2 cellular entry occurs upon binding of its Spike protein to angiotensin-converting enzyme 2 (ACE-2) and TMPRSS2 receptors [7]. To enable the use of cell lines with comparably low physiological levels of human ACE-2 and TMPRSS2 that SARS-CoV-2 does not infect efficiently, overexpression of entry receptors can be used to enable efficient replication. For example, overexpression of human ACE2 (hACE2) or TMPRSS2 on the lung adenocarcinoma cell line A549 permits infection with SARS-CoV-2 [106]. Additionally, culturing A549 cells in an air-liquid interface (ALI) culture increases the endogenous expression levels of ACE2 and TMPRSS2 following adaptation to culture conditions [107]. Similarly, the overexpression of ACE2 on Hela (cervical cancer cells) enables efficient SARS-CoV-2 entry [108] as previously shown for SARS-CoV [109].

For monoclonal antibodies, it has been shown that the overexpression of receptors skews the antiviral activity significantly, and complicates the interpretation of results [110] Specifically for the assessment of entry inhibitors, the roundtable recommends that cell lines with physiological levels of receptor expression should be preferred for the assessment of antiviral activity.

Commonly used Tier 2 Primary cell models.

Following the identification of a small set of optimised leads or a lead candidate, compounds may be evaluated in more physiologically relevant Tier 2 assays. For SARS-CoV-2, these can include human airway epithelial cells, normal human bronchial epithelial cells or iPSC derived pneumocytes. Primary cells may offer a more physiological immune system compared to cancer cell lines commonly used for Tier 1 assays, which can be particularly relevant for the assessment of molecules interacting with the host-immune response [111–113]. In addition, primary cell models allow for a more translationally relevant understanding of drug uptake and cellular metabolism. In documented SARS-CoV-2 drug discovery efforts to date, data from both Tier 1 and Tier 2 assays have been used for human dose predictions [25,114], with the alignment of Tier 2 with Tier 1 assays results explicitly noted if chosen.

However, primary cells are generally not used for screening compounds as cells are expensive to source, more difficult to culture and scale, and assays may show high variability. For example, human airway epithelial cells and normal human bronchial epithelial cells have to grow in an air-liquid interface with differential treatment on the basal and apical cell sides [115,116]. In addition, the phenotype of primary cells can only be maintained for a short number of passages limiting their utility for high-throughput set-ups [117], as they can rapidly de-differentiate and adopt senescence phenotypes [118]. These limitations also apply for more complex cellular models such as bio-printed and 3D organoid models, which currently do not play any role in routine antiviral drug discovery [119].

Infecting Strains

A second variable in setting up in vitro antiviral assays for drug discovery includes the choice of the infecting virus. Some viruses such as SARS-CoV-2 replicate readily in numerous human and animal cell lines, for others such as Hepatitis C virus this can pose a significant scientific challenge [120].

Based on previous experimental knowledge from SARS-CoV, it was rapidly established that various clinical SARS-CoV-2 isolates readily infect a wide range of human and animal cell lines [105]. Significant variability in replication efficacy has been reported for different SARS-CoV-2 strains, with the Delta variant showing increased pathogenicity [121], and Omicron demonstrating differential viral kinetics with significantly longer replication cycles compared to WT SARS-CoV-2 [122,123]. In addition, differential dependency on entry receptors has been suggested, with SARS-CoV-2 Omicron BA.1 showing higher relative affinity to ACE-2 whilst SARS-CoV-2 Delta depends on high levels of TRMPSS2 expression for viral entry [16]. Therefore, it can be challenging to compare antiviral efficacy in cellular assays against multiple SARS-CoV-2 variants.

In addition to the variable pathogenicity of SARS-CoV-2 variants, it has been shown that SARS-CoV-2 can adapt to different cell lines through the accumulation of viral mutations upon viral passaging. These viral adaptations can render additional cell lines as susceptible to the virus such as liver cell lines (Huh7 and Huh 7.5) and lung cancer lines (unmodified Calu-1 and A549) [124]. In addition to naturally circulating viral strains, synthetically engineered viruses such as infectious cDNA clones and reporter viruses can be used in cell

culture and optimised to express engineered molecular markers facilitating high-throughput screening [125–127].

Non-infectious cellular in vitro models with replicons.

Non-infectious subgenomic replicons can be used to connect enzymatic assays and cellular systems. Subgenomic replicons are artificially constructed RNA molecules containing all of the viral genome except genes that encode the structural proteins. They can replicate in cells, but are unable to infect other cells, thus are safer to operate and can often be handled in a BSL2 laboratory environment. Replicon systems provides a cell-based assay system to interrogate the fitness of different protein mutants, as well as screening potential antivirals. Historically, replicon systems have been crucial in drug discovery for viruses where cellular replication was difficult to achieve such as HCV, or where laboratory handling is associated with significant health risks [128–130].

For SARS-CoV-2, several replicon systems have been reported. In principle, these are based on the deletion of selected viral proteins (S, E and/or M proteins) and the addition of genes encoding firefly luciferases (Luc), green fluorescence (GFP) fusion proteins, or others [131–133]. SARS-CoV-2 replicons suitable for BSL2 environments can rely on a variety of technologies, including (1) transient reporter replicons [134]; (2) trans-complementation systems [135], and (3) attenuated viruses with deletions of viral accessory genes [136].

However, replicons may have limitations; (i) they cannot be used for the assessment of targets that are not included in the construct, (ii) interdependency of host and viral targets and immunological responses may not be modelled, and (iii) compounds optimised against a replicon may not show activity against wild-type virus, so should always be cross-checked against virus during development. In addition, with increasing availability of high-throughput SARS-CoV-2 BSL3 screening facilities [137–139] the future scientific role of SARS-CoV-2 replicon systems replacing antiviral assays as a screening option remains an open question.

Other experimental factors

Choosing a high-throughput reliable readout makes an assay usable for drug discovery efforts. Scalable and automatable readouts that can be reproducibly quantified and may be run in a multi-plate format (such immunofluorescence or cytopathic effect assays) lend themselves for high-throughput set-ups, whereas plaque assays come with a high experimental work-load and show high variability between runs. Additional factors that can impact on the comparability of assay results include assay timings (time of infection and treatment time), the amount of protein used in the assay (especially with highly protein-bound compounds), as well as compound dilution and handling.

In summary, we argue that the availability of high-throughput reproducible assays, rather than those with physiological readout, remains of utmost importance to small molecule drug discovery efforts for most directly acting antivirals. Further, in a pandemic situation with urgency in mind, even the nomination of development candidates for pre-clinical

development may be executed solely based on Tier 1 cellular assays. Compound specific considerations such as metabolic conversion into active metabolites and high affinity to efflux pumps determine the choice and experimental set-up of the cell culture model. Our collective learnings are that once a relevant model for a series is established, there is limited value in comparing across different assays and cell lines due to high assay variability, since the most useful readout is within an assay and comparing across different compounds to inform SAR and progress lead compounds. For pandemic preparedness, it will be crucial to develop and openly share assays and reagents for viruses of pandemic concern, in the hope of translating scientific understanding and facilitating comparisons across antiviral drug discovery efforts.

Animal and human models

Animal models

The first wave of directly acting antivirals against SARS-CoV-2 were advanced solely based on in-vitro cellular data and projection for the human exposure to exceed a certain level sufficient for a pharmacodynamic effect. We suggest that it is sufficient to define a cellular EC90 in a primary cell model and determine the human dose and dosing frequency to remain above the cellular benchmark at all times (e.g. serum protein unbound free fraction of drug at Cmin > EC90 for treatment duration). We note that although efficacy in animal models is a generally recommended (but not an absolute requirement) for regulatory approval of human therapeutics, approval of antivirals without going through animal models was precedent for HIV and HCV.

Current animal models for SARS-CoV-2

For SARS-CoV-2 infection, a range of animal models have been described [140] and extensively reviewed [141,142]. Based on animal models described for SARS1 [143,144], both ferret and [145,146] hamster models [147,148] that are susceptible to wild-type SARS-CoV-2 infection were rapidly deployed early in 2020 and 2021. However, both models come with significant logistical and financial overheads. More recent studies utilise mouse models which are easier to deploy due to their high accessibility, low cost, rapid breeding speed, and ease of manipulation. For SARS-CoV-2 infection in mice, mouse models expressing specific SARS-CoV-2 entry receptors (e.g. K18-hACE2 overexpressing mice) infected with wild-type virus [149], or wild-type mice infected with mouse-adapted viral strains [150] have been frequently used. In addition, the acquisition of the 501Y mutation in variants of concern (VOC) has been shown to enable the infection of wild-type mice and other rodents, particularly in aged animals, albeit at overall lower viral loads [151]. Pre-clinical studies in non-human primates have been predictive of COVID vaccine outcomes in clinical efficacy studies [152], but are not routinely used in small molecule drug discovery.

Similarly to human disease, animal models for SARS-CoV-2 may show major differences in viral load and pathology dependent on infecting viral strain [153]. Specifically, animal susceptibility appears to be linked to the affinity of the SARS-CoV-2 spike protein to the ACE-2 receptors, and variant-specific spike substitutions such as N501Y, D614G, and V367F impact on transmission in animals [152]. Significant variant-specific differences in

viral load distribution have been described, with lower viral loads and little weight loss observed for Omicron compared to Delta in wild-type and hACE2-transgenic Syrian hamsters [154], in line with clinical data available suggesting that omicron does not have higher viral loads in humans [155,156]; as well as attenuated disease pathology for the Omicron variant in animals [154,157–160].

Overall, variant-to-variant differences in both animal and humans are expected to lead to time lags in establishing the translatability of new animal models with every new variant of concern. Therefore, firmly establishing the translational relevance of an animal model may not be significantly less arduous than performing clinical studies in patients, limiting the translational use of animal efficacy models.

What can animal models add?

The panel agrees that animal efficacy models should not be on the critical path of small molecule antiviral discovery against SARS-CoV-2. However, for small molecule drug discovery of directly acting antivirals, animal models can give additional reassurance if investigated inhibitors impact significantly on SARS-CoV-2 viral load and histopathological endpoints. This is especially true if dose-related effects on viral load or “mouse health” (e.g. weight or lung function assessed by whole body plethysmography) can be linked directly to target coverage based on unbound drug exposure [38,98,161,162].

Animal models do have the potential to play an important role for pandemic preparedness: This is particularly relevant for viral diseases such as Ebola, where a human Phase 2a study is not possible as no human disease is circulating at the time or challenge models are not available [163–165]. In these cases, animal efficacy in a relevant model, in combination with human PK and standard safety studies, may be sufficient for drug approval [166], to ensure that a compound is available to be rapidly deployed in the case of a pandemic threat.

In addition to these considerations, data from infected animals may provide additional insights into addressing questions that are not directly linked to the discovery of directly acting antivirals. Despite potential differences in pathology between animal and human models, these may include investigations on the prevention of transmissibility of disease and the impact of viral load on transmission [116], effect of age and comorbidities on disease progression [167], organ and brain involvement, vascular symptoms, co-infections and secondary bacterial infections, “long COVID” [168], immunological sequelae [169] etc. However, for most of these presentations, the human pathogenicity remains unclear and many existing animal models for SARS-CoV-2 are not yet validated [152].

Human challenge models

Ultimately, the most relevant model to assess natural infection, viral load distribution and efficacy of antiviral inhibitors is a human viral challenge (HVC) model. This model enables the assessment of viral load kinetics in human healthy volunteers, and comes with the unique opportunity to follow the viral life cycle, with certainty of the time of infection, assessment of viral shedding and prospective assessment of symptoms [170].

Human viral challenges have been used previously to assess the natural cause of acute respiratory viruses, including Respiratory syncytial virus (RSV), Influenza, human rhinovirus, and most recently COVID [171,172]. Generally, the availability of “rescue” treatments and up-front knowledge on potential human disease pathology and complications are paramount to the conduct of HVC. Nevertheless, for novel human pathogens such as SARS-CoV-2 with incomplete understanding of long-term disease implications, complex ethical issues remain that have to be carefully assessed before embarking on HVCs.

Potential confounding factors in HVC are the usually chosen narrow time between infection and treatment start, which can be precisely controlled in HVC but is less controllable in natural infection, potentially leading to an overestimation in compound efficacy. The careful selection of healthy volunteers and regular sampling may also explain some of the differences noted between natural infection and HVC trials for SARS-CoV-2 and other respiratory viruses [172], for example higher peak viral loads measured for SARS-CoV-2 HVCs [172,173], and more common upper respiratory tract infections in RSV HVCs, rather than lower respiratory tract infections in natural infection [174]. Overall, data generated in HVC studies may be highly variable dependent on inoculation dose, the viral strain used and immune profile and age of the healthy volunteers.

A key point where human challenge models can contribute to clinical decision making is the determination of treatment duration based on the assessment of human viral load evolution, especially in comparison with a non-treated control group. This is enabled by an objective assessment of viral circulation clearance with regular viral load measurements after a known infection using a standardised infection dose, which allows for “back-filling” the target product profile including treatment duration, as previously shown for influenza infection [175]. However, translation from efficacy in human challenge study to natural infection is not certain: for Ruprintrivir, an investigational protease inhibitor against human rhinovirus, efficacy in human challenge studies overestimated the utility for the natural infection [18,176].

Preempting resistance

Resistance mutations can render an antiviral therapy inefficient. Viral mutations can occur spontaneously (especially in rapidly mutating viruses), and are selected to preferentially replicate under immunological pressure or upon selection pressure exerted by drug therapy. Nonetheless, only mutants that are transmissible and cause adverse pathologies are of concern.

The likelihood of a virus developing mutations depends on (i) viral factors, e.g. how readily does the virus mutates overall (does it have a polymerase proofreading function), (ii) host factors including the immunological pressure (e.g. is the infected patient immunosuppressed) and (iii) environmental factors such as antiviral treatment - the chosen drug target, whether the treatment is given as a single or combination therapy, and whether it is given for an acute or chronic viral infection. Drug-induced resistance has been observed across the spectrum of antiviral therapeutics and is largely independent of treatment duration, from short courses for acute infection e.g. 5 days of Oseltamivir [177] or even

single dose treatment with Baloxavir [178], to longer treatment courses used for chronic viruses [179].

Even though the lower likelihood of viral drug resistance is commonly used as an argument for host-targeting antivirals, this is not necessarily a solution to addressing resistance mutations. For example, targeting cyclophilin with small molecule inhibitors caused mutations in the HCV NS5a protein [180], and targeting CCR5 caused mutations in the HIV gp120 protein [181,182].

The roundtable suggests that the essential strategies to circumvent drug-induced resistance are designing compounds which sit tightly in the “substrate envelope” of the binding site, driving the safe free drug concentration as high as possible, and considering combination therapy. At the discovery stage, several approaches exist to preempt the development of resistance.

Selecting the target

Sequence conservation

A commonly used approach to identify viral targets that carry a low tolerance to resistance mutations is selecting targets based on sequence conservation [14,183–185]. Different levels can be considered, such as sequence conservation across different families of the viral family *coronaviridae* (across alphacoronaviruses [e.g. 229E, NL63] and betacoronaviruses [e.g. SARS, SARS-CoV-2, OC43], or circulating variants within SARS-CoV-2 (e.g. alpha, beta, omicron). For selected targets such as the polymerase, it is even feasible to consider targeting strategies across other viral families, such as for Filoviridae (e.g. Ebola) and Togaviridae (e.g. Venezuelan equine encephalitis virus) [89].

Interpreting drug resistance mutations occurring in patient populations and during clinical trials

Known data on resistance mutations identified through sequence surveillance in SARS-CoV-2 infected patients can feed into the target selection. This is particularly relevant if there is target-specific evidence of drug-induced resistance, as described for early protease inhibitors developed for the treatment of HIV and HCV infections. Of note, coronaviruses overall accumulate less mutations than other RNA viruses such as HIV and HCV [186], where multitudes of quasispecies can be detected in each infected individual, likely due to the lack of proofreading functions of the viral RdRps [187–189]. In contrast, coronaviruses possess a unique error-correcting function that had been unknown among RNA viruses prior to its discovery in SARS-1 [190], with nsp14 excising nucleotides misincorporated by the low-fidelity RdRp and thereby lowering the replication error rate in comparison to that of other RNA viruses [191–193].

When interpreting resistance mutations in patients treated with directly acting antivirals and phylogenetic data, several caveats have to be taken into account:

Firstly, natural mutations occur without drug pressure (surveillance of circulating virus), and do not imply pre-existing drug resistance for a certain target, e.g. baseline resistance-associated mutations do not predict treatment failure [194].

Secondly, variants need to persist and remain transmissible in order for them to become relevant resistance-associated variants (RAVs). Viruses with RAVs in their genome often have a fitness cost or growth disadvantage compared to the wild type virus in the absence of selective pressure. Evolutionary competition between wild type virus and mutants has been observed in patients, where variants rapidly expand in the presence of selection pressure which preferentially suppresses wild type virus replication, but once the drug is removed the wild type virus outcompetes the virus with RAVs mutants again, for example in HCV [194]. Nevertheless, several known drug induced variants are fit and transmissible, such as those described in influenza against neuraminidase inhibitors [195,196] and cap-dependent endonuclease inhibitors [197–199].

Finally, even observed mutations under treatment do not per se imply drug-induced escape. This is particularly important when interpreting data from clinical case reports [200–202]. Large-scale clinical studies that sequence the whole viral genome and monitor viral load longitudinally across the treatment duration for a patient cohort are needed to identify causative drug-induced mutations (e.g. the PANORAMIC trial [203]). Even if the mutant is replication competent and persists, the impact on disease presentation and progression (e.g. mortality, hospital admission, or viral load progression) has to be assessed on a large scale.

Drugging the target

Strategic approaches to drugging the target can mitigate resistance development at the discovery stage. Common approaches employed by us are: (1) Structural biology - defining resistant-robust regions of the protein to target. (2) Enzymology - configuring biochemical assay cascades to screen for resistant-robust antivirals. (3) Cell culture systems - designing cellular antiviral experiments that can shed light on propensity for resistance development.

Structural biology

A structure-based discovery approach allows the medicinal chemistry campaign to focus not only on maximising protein-ligand interaction, but also defining the region of the protein to target with chemical inhibitors. In particular, recent advances in cryo-EM allows for rapid determination of complex structures [204–206]. To circumvent viral resistance mutations from the start, several levels have to be considered, including what sequence to base the drug discovery effort on, what site to drug and how to drug the active site.

Sequence selection

The first stage in a structure-based approach is selecting the relevant sequence to crystallise. This is both a pragmatic consideration, as even single mutations can affect the propensity to form crystals, but also important for the analysis of resistance. Common approaches include selecting a wild-type sequence, a clinically relevant strain, or a consensus sequence, i.e. comprising the most frequently occurring residues across variants.

Active vs allosteric site

The consensus within this round table is that it is preferable to directly target the enzyme active site, mainly based on data generated in HCV and influenza. In contrast, allosteric inhibitors may have a lower barrier to resistance. As demonstrated by non nucleoside NS5B inhibitors developed for HCV where allosteric sites are highly polymorphic, these were associated with a lower barrier to resistance in comparison to nucleoside inhibitors targeting the active site [207,208]. Another example is influenza cap-dependent endonuclease, where Baloxavir targets a distal pocket in the active site [209] and is known to rapidly generate treatment-emergent resistance mutations [178].

Substrate envelope

The substrate envelope concept hypothesizes that within the active site, a high barrier to resistance can be achieved by designing compact inhibitors that stay tightly within the *substrate envelope*. This is defined as the space spanned by a key set of residues that interact with diverse native substrate sequences [210,211]. Mutations within the substrate envelope are likely to cause a significant disruption in enzyme function, whereas mutations outside the envelope may incur significantly less fitness cost. The substrate envelope approach can be further refined by designing the inhibitor such that it interacts predominantly with consensus residues, within the substrate envelope, that are shared across a viral family, e.g. coronaviruses [40].

This concept has been developed and validated for HIV and HCV protease inhibitors [212,213], and recently deployed in SARS-CoV-2 [214]. For HIV, analysis of structures protein-ligand complexes of FDA-approved protease inhibitors show that inhibitor-residue contacts outside the substrate envelope correspond to the known primary drug resistance mutation sites [212]. For SARS-CoV-2, recent work defines the substrate envelope of Mpro, by crystalizing a library of substrate peptides in native cleavage site [214]. Analysis of several specific SARS-CoV-2 protease inhibitors reveal that the inhibitors mostly lie within the substrate binding envelope, though some parts of the molecules interact with residues outside the envelope. However, without comparative in-vitro studies with SARS-CoV-2 MPro inhibitors engaging different regions of the substrate envelope, or ultimately comprehensive sequencing data on patients treated with SARS-CoV-2 MPro inhibitors, it is too early to tell whether these interactions will manifest as clinically observed resistance mutations.

Enzymology

Configuring an appropriate assay cascade

Using enzymology, the impact of selected viral mutation on enzyme function can be defined. Broadening this approach, saturation mutagenesis provides a comprehensive assessment of enzyme function. For example, a recent study mutated each amino acid for SARS-CoV-2 Mpro to reveal which mutations still result in a functional enzyme [215]. Reassuringly, the saturation mutagenesis approach shows that mutationally intolerant residues are also conserved across homologues. In addition to the active site, additional mutationally intolerant sites were revealed at the dimer interface and allosteric communication network

between the active site and the dimer interface. However, further work to interrogate the fitness of these mutant enzymes in authentic viral systems using reverse genetics is needed. We note that a similar mutagenesis approach has been successfully applied to predict clinically relevant patterns of resistance in bacterial beta-lactamase [216,217].

In addition to assessing variants within SARS-CoV-2, further robustness can be engineered by designing inhibitors that are active against enzymes from different viruses within the same family, an approach that has been used by panel members in internal programs. However, routinely screening compounds against a broad panel of enzymes as primary screens is laborious and expensive. An alternative approach could be deploying a "consensus enzyme" building on the enzyme engineering literature [218,219], whereby a single model enzyme displaying the most frequent residue across viruses within the same family is designed.

Overall, as understanding about circulating variants and clinical drug resistance against a target class emerges, the screening cascade should include enzymatic assays of circulating variants or clinically observed drug-resistant mutations. However, in the context of a drug design campaign that races against an emerging and evolving pandemic, constantly moving goalposts by including new variants in the assay cascade are not feasible.

Degrader Paradigm

Beyond direct enzyme inhibition, the roundtable identifies the degrader paradigm as another potentially viable way to overcome resistance. This approach exploits intracellular proteolysis to break down the target protein. The paradigmatic example is Proteolysis-Targeting Chimera, PROTACs, which are heterobifunctional molecules where one end binds to the target protein, and the other end engages with a ubiquitin ligase, resulting in ubiquitination and subsequent degradation by the proteasome.

Potential advantages of PROTACS are that (i) lower affinity binders can have a biological effect, as the target protein is irreversibly removed, (ii) degradation by PROTACs is catalytic as the ligand is not consumed, (iii) the pharmacodynamic efficacy is driven by the viral protein production rate, and can extend beyond the detectable pharmacokinetic presence of the PROTAC molecule. The feasibility of a PROTAC has been explored for the HCV protease [220], and recent work demonstrated the feasibility of a degrader approach to target SARS-CoV-2 RNA [221].

Cell culture systems

Assessing resistance with replicons

Non-infectious replicon systems can be modified to assess the effect of resistance mutations on viral replication and the efficacy of antiviral compounds. The field of replicon and drug resistance in SARS-CoV-2 is still nascent. However, in HCV, a large body of literature has elucidated mechanisms of resistant replicon formation against protease inhibitors [222,223], polymerase inhibitors [224,225] and combinations [226], as well as comparing the barrier of resistance of nucleoside and non-nucleoside inhibitors [227].

From a drug design perspective, inhibitors can be designed to display a favourable profile against a panel of replicons harbouring mutations from different viral genotypes, or known drug-resistant mutations observed after treatment with other inhibitors from the same class, as reported for HCV [228,229]. Further, the fitness cost of mutations can be quantified by the replication efficiency of a replicon system [222]. Of note, mutations with efficient growth in a replicon may not be fit in vivo [230], thus careful validation of in vitro-in vivo correlations and comparison with clinically observed mutations [231] are required to shed light on the fitness cost of resistance.

Serial passaging of infectious virus

Beyond replicons, cell culture systems with live virus can be employed to model resistance development under drug pressure. A typical experiment is serial passaging - subjecting the virus to low concentrations of the inhibitor over long periods of time, isolating and growing the surviving mutants, then re-exposing these to another cycle inhibitor treatment. The number of cycles required for resistant mutants to emerge is a metric that assesses the barrier to resistance. The replicative fitness of the mutants can be quantified by measuring the growth rate of the mutant, as well as cellular competition studies where mutants are co-infected with the wild-type. Further, sequencing of viral mutants can reveal residues that are susceptible to mutations in cell culture. In cases where the chemical compound is discovered phenotypically, serial passaging studies can help to elucidate the viral target [232,233].

Viral passaging can be used during lead optimization, where the barrier to resistance may be used as an additional factor to select the lead series [234]. Overall, significant series-specific differences in barrier to resistance are common, as different chemical series typically contact different sets of residues in the binding site. Passaging experiments have been performed using remdesivir in the SARS-CoV-2 related virus Mouse Hepatitis Virus (MHV) [235], and later for SARS-CoV-2 [236,237], revealing potential point mutations that may confer resistance. Viral passaging on the main protease inhibitor nirmatrelvir was also performed in MHV to study the potential sites of mutation and degree of reduction in nirmatrelvir sensitivity [39]. Additional mutations were reported for serial passaging of probe compound ALG-097161 leading to a combination in mutations that also confer resistance to nirmatrelvir in vitro [238]. However, the relevance of these mutations detected in serial passaging experiments has to be independently verified by clinical trial results.

Nonetheless, serial passaging studies of clinical approved inhibitors remains a contentious issue due to the potential of selecting for resistant variants, which may impact biosecurity and public health. As such, funding agencies have put in place restrictions for such studies [239].

Deploying the therapeutic

Strategic approaches to preempt resistance development can also be used at the clinical stage.

First, combination therapies comprising antivirals targeting multiple targets have a lower likelihood of selecting for escape mutants [240,241], and are commonly used in clinical settings for a variety of viruses including HIV and HCV. To date, only influenza and HSV

have monotherapies in routine clinical use, whilst optimal treatment regimens for SARS-CoV-2 are being explored. Further, it is likely that certain patient populations, such as immunosuppressed patients, may require combination therapy as they are more likely to harbour resistant viruses.

Second, maintaining high drug concentrations (with minimal free drug concentrations as high multiples of EC90) above may lower the likelihood of inducing viral resistance. For HCV direct acting antivirals, the consistency of EC90 coverage (here correlating with treatment adherence) has been linked to the emergence of resistance mutations [242]. For SARS-CoV-2, the appropriate level of cover over EC90 is not yet clear, with early clinical data emerging: The reported clinical strategy for Nirmatrelvir and PBI-0451 are selecting dosing that enables Cmin > EC90 for 90% of patient population [243] [40]. For S-217622 (Ensitrelvir), less stringent criteria of Cmin > EC50 were reported for animal efficacy [25], whilst pre-clinical data on EDP-235 suggests that 5-25x EC90 coverage is the aim [244]. This roundtable argues that a pragmatic approach, driving the medicinal chemistry campaigns to increase the coverage as much as possible, whilst recognising that target coverage may be a point of product differentiation (c.f. **Table 3** for ideal vs pandemic Target Product Profile).

Discussions and Conclusion

The exigencies of the COVID-19 pandemic have spurred a wave of rapid drug discovery campaigns. In less than 2 years, several antivirals were discovered, clinically evaluated, and approved. In this article, we summarised the key scientific drivers and considerations behind these sprint discovery campaigns, and how knowledge from previous antiviral drug discovery campaigns were fruitfully deployed. Beyond scientific approaches, our experience suggests that the broader organisational context has enabled rapid discovery, outlining future directions for the community.

A common strand underlying many campaigns is the large degree of optimism amongst the project team, and buy-in from all levels, from senior leadership to team members. Planning and executing future experiments at-risk assuming previous experiments will be successful, whilst also having a mitigating plan in place in case of failure, is critical to moving fast. Further, as drug discovery is inherently risky, management buy-in and upfront investment is needed to move fast. In the fast-moving research environment of a pandemic, it remains an open question how public sector funding, traditionally more risk-averse, can rapidly enable drug discovery.

Another fruitful experiment during the pandemic was the unprecedentedly collaborative philosophy across biopharma, nucleating public-private partnerships such as IMI CARE [245], close-knit consortia of pharma [246], and even completely open science consortia with biopharma participation [26]. Further, the rapid implementation of clinical trial networks such as ACTIV, RECOVERY and PANORAMIC [8,9,247–249] has greatly contributed to the accelerated assessment of potential COVID-19 therapies. The unique severity of the COVID pandemic – 6.3M deaths to date and a global shut down during the height of the pandemic – created the strong impetus for these collaborations. The successes of these projects can inspire more openness and collaboration in the biopharma industry, and the recognition that a rising tide lifts all boats, motivated by the drive to prevent future pandemics. We believe

commercial organisations should be more willing to work quickly, closely, and collaboratively together when there is collective harm happening.

Further, the pandemic has been an exceptional case for fast-moving and rigorous regulatory emergency review and approval, with agencies establishing rapid response mechanisms as early as May 2020 [250,251]. Proposed mechanisms outline more efficient processes to receive rapid agency feedback on supporting data with the primary aim of enabling clinical trials, alongside a detailed definition of clinical endpoints [252]. The roundtable argues that in view of the rapidly changing clinical picture with increasing natural immunity and vaccination rates, as well as variable pathogenicity of circulating strains, a regular review of acceptable clinical trial endpoints should be conducted. Those initially used for small molecule clinical trials in COVID-19, such as mortality and hospitalization rate, are now considered too low in frequency to support reasonable sized clinical trials. Instead, it may be warranted to consider additional primary endpoints like viral replication, which are easier to standardise compared to symptom-related endpoints, and considered acceptable for other viral diseases such as HIV and HCV [253–255]. Especially viral kinetic data from human challenge trials, not immediately available at the start of the pandemic, may feed into defining alternative endpoints.

Ultimately, a successful drug discovery campaign is contingent upon selecting and drugging the right protein target. We note that mechanism-free repurposing has been widely attempted during the COVID-19 pandemic, but failed to deliver therapeutics [256]. Early positive results were shown to be due to confounding nuisance factors such as phospholipidosis [257]. This is perhaps not surprising, as most therapeutics and chemical compound libraries are optimised for human targets, which are generally dissimilar to viral targets.

Therefore, we conclude with a call to arms on pandemic preparedness. A responsive mode to antiviral drug discovery, even armed with modern technology, will be too slow to prevent the significant human and economic catastrophe that a fast-moving pandemic causes - it took a decade to offer a therapeutic in HIV and about two years for COVID. With significant funding in place, for example the recent announcement by the National Institutes of Health allocating $577 million to fund 9 Antiviral Drug Discovery (AViDD) Centers for Pathogens of Pandemic Concern [258], the question becomes how do we best nucleate a concerted effort for pandemic preparedness?

One strategy is systematically targeting the viral proteome. Out of the 16 non-structural proteins and 4 structural proteins in SARS-CoV-2, to date only 3 (Mpro, PLpro and RdRp) have validated chemical probes. Once these existing targets are drugged and resistance inevitably emerges, we will need to uncover new viable viral targets or new mechanisms of action or judicious combinations of therapeutics with no cross-resistance. Another related strategy is open dissemination of tools such as assay protocols, building on successful precedents for human targets such as the RAS initiative [259] and the Structural Genomics Consortium [260]. Focused efforts should be invested into ensuring the myriad of assays developed, and resulting data, are disseminated with good data management practices, to ensure Findability, Accessibility, Interoperability and Reusability [261].

Considering the human and economic toll of a pandemic, we argue that public sector investment should be put into systematically developing chemical probes and focused

libraries against proteins in viruses of pandemic concern. Concerted funding should be in place to push chemical matter against promising targets into early clinical development, so that it can enter Phase 2 clinical trials when a pandemic of the same viral family strikes. This is even more pressing for other viruses of pandemic concern, such as Dengue and Zika, which are endemic in the Global South and currently lacking effective therapeutics. Thus a global health angle to drug discovery and development is acutely needed to alleviate significant current unmet medical need.

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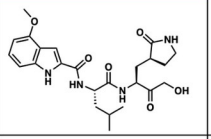
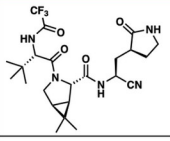
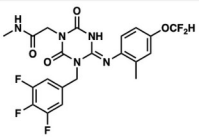
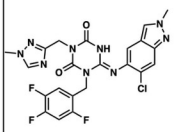
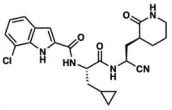
Competing interests

All authors and/or their employers are involved in COVID drug discovery or development programs, and may continue to be involved in future programs. These programs may lead to the generation of intellectual property for their respective employers, and may directly or indirectly contribute to past, present or future revenue of their respective employers. MDH is an employee of NCATS, AK is an employee of Pardes Biosciences, LAP is an employee of Vir Biotechnology, KSS is an employee of Takeda California Inc, US is an employee of Gilead Sciences, JAT is an employee of Novartis Institutes for Biomedical Research, AVD is an employee of Oxford University, and consults for PostEra AI, AL is an employee of Cambridge University and CSO for PostEra AI.

	NSP5	NSP12			NSP3	NSP3	NSP13		NSP14		NSP15	NSP16
	Mpro	Polymerase			PLPro	Mac1	Helicase		Methyltransferase		Endouribo-nuclease	Methyltransferase
Sequence conservation												
	catalytic site	catalytic site	ectopic site	NIRAN ADP site	catalytic site	catalytic site	ATPase site	central channel	catalytic site	ectopic site	catalytic site	catalytic site
Sequence conservation (Coronaviruses "Pocketome")	52%	94%	50%	87%	29%	62%	71%	92%	70%	61%	78%	61%
Chemical probe validation												
Structural/Fragments/HTS data	✓		✓		✓	✓	✓		✓		✓	✓
Availability of chemical probes	✓		✓		✓		✓		✓			
Mechanism of action validation												
Biological evidence (other viral families) MoA confirmed in vitro/in vivo	✓		✓				✓				✓	
Biological evidence (coronaviruses) MoA confirmed in vitro/in vivo	✓		✓		✓							
Clinical evidence (other viral families)	✓		✓				✓				✓	
Clinical evidence (Coronaviruses)	✓		✓									

Table 1: Summary of mechanism of action validation including clinical evidence, chemical probe validation, structural data and sequence conservation across 27 α- and β-coronaviruses for selected SARS-CoV-2 targets (data taken from ref** [14]**, which considered sequences up to 7/31/2020).

Main protease inhibitors

Compound name	Hit finding strategy	Starting point	Clinical candidate structure	HLM CL_{int} (µl/min/mg)	Rat CL_p (ml/min/kg)	Rat oral F (%)	SARS-CoV-2 antiviral activity (EC_{50}/nM)	Clinical stage
Nirmatrelvir	Lead from historic SARS-CoV campaign			24.5	27.2	50	62 (human airway epithelial cell)	EUA
S-217622	Structure-based virtual screening and pharmacophore filtering			97% after 30 min of incubation	7.3	111	370 (VeroE6/TMEPRSS2, CPE)	Phase 3 NCT05305547
PBI-0451	Historic data from patents and docking across available Mpro structures	Undisclosed		Undisclosed	Undisclosed	Undisclosed	32 (WA1 strain, iPS-AT2, PFU)	Phase 1 NCT05011812

Polymerase inhibitors

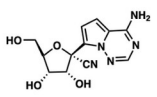
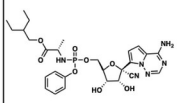
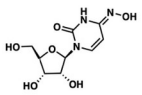
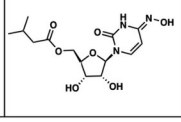
Compound name	Hit finding strategy	Starting point	Clinical candidate structure	HLM CL_{int} (µl/min/mg)	Rat CL_p (ml/min/kg)	Rat oral F (%)	SARS-CoV-2 antiviral activity (EC_{50}/nM)	Clinical stage
Remdesivir	Designed as a RNA virus antiviral. Hit obtained from screening a nucleoside library			Undisclosed	Undisclosed	i.v. drug	9.9 (human airway epithelial cell)	Approved
Molnupiravir	Designed as a RNA virus antiviral.			Undisclosed	Undisclosed	52	670-2660 (A549), 320-2030 (VeroE6)	EUA

Table 2: Main protease and polymerase inhibitors in clinical use or late stage clinical trials. *Data sourced from the primary medicinal chemistry literature or fact sheets released by the FDA or EMA. Nirmatrelvir (Pfizer Inc, ADME/PK: [38] , antiviral: [39]), S-217622 (Shionogi, ADME/PK and antiviral: [25]), PB1-0451 (Pardes Biosciences, antiviral: [40]), Remdesivir (Gilead Sciences, antiviral: [41]), Molnupiravir (Merck, antiviral: [42], rat oral bioavailability: [43])*

TPP	Pandemic response	Ideal therapeutic
Route	Orally bioavailable	Orally bioavailable
Dosing	Three times a day	Once, or once a day
Dose	up to 1g x 3 times daily	<250mg
PK/PD	Cmin>1xEC90, unbound	Higher coverage – correlating with high barriers to resistance
Drug-drug interactions	Acceptable	No
Co-dosing with PK-enhancers	Acceptable	No
Patient cohorts	Narrow cohort acceptable	Available to all (e.g. women of child-bearing age)
Therapeutic window	Within 48 hours of infection	Extended up to 5 days

Table 3: Example of Target Product Profile for an acute viral infection, for both a therapeutic developed for immediate pandemic response and potential next generation "ideal" therapeutics. The TPP can be used as a framework to evaluate both repositioned compounds as well as novel therapeutics.

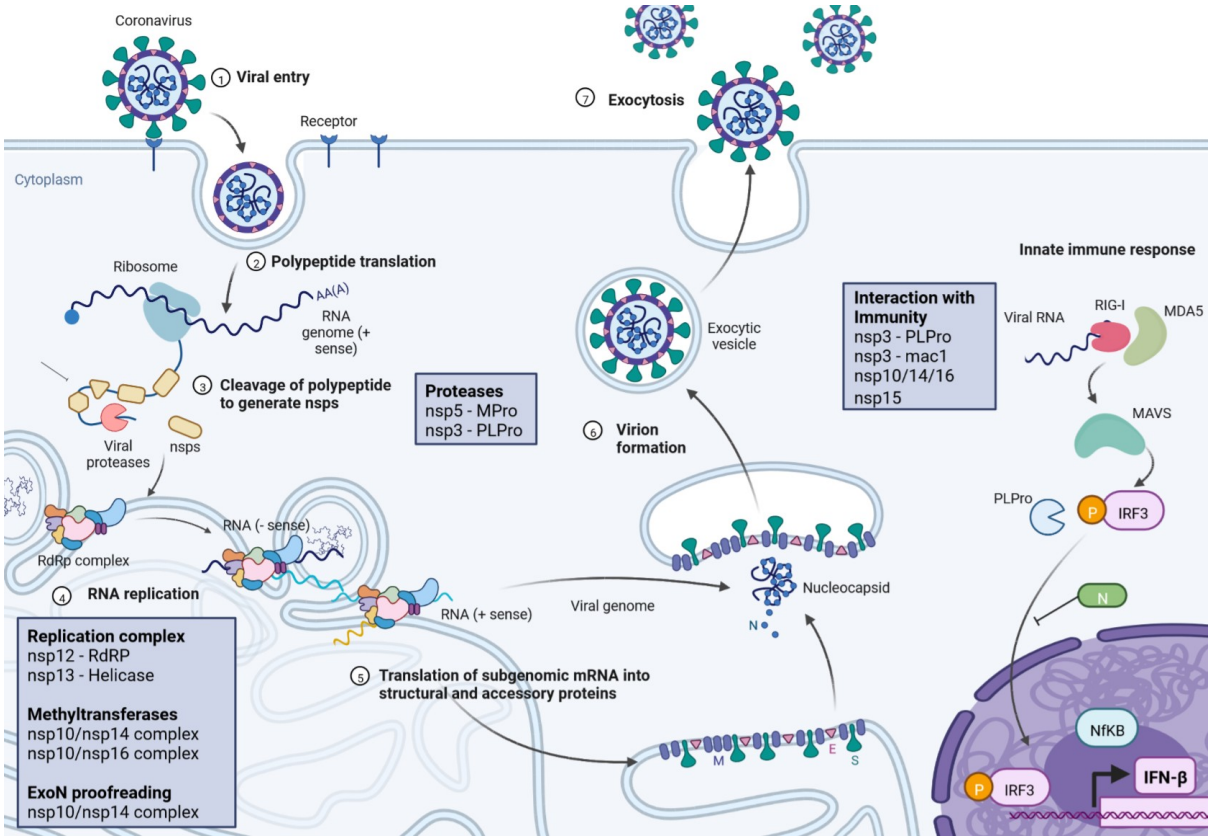


Figure 1: Key protein targets in the SARS-CoV-2 replication cycle. *(1) Upon binding of SARS-CoV-2 virus to extracellular receptors angiotensin-converting enzyme 2 (ACE-2) and, depending on SARS-CoV-2 viral strain to the cell surface serine protease transmembrane protease serine 2 (TMPRSS2) that promotes viral uptake [16], the virus enters the cell. (2) Following uncoating and release of the viral RNA the incoming genomic RNA is translated into two large open reading frames (ORF1a and ORF1b). (3) These are co- and post-translationally processed by viral proteases into non-structural proteins (nsps) that form the viral replication complex. Continuous cleavage of the polyprotein is required for sustained RNA synthesis, suggesting that formation of the replication complex is dynamic and must occur continually. (4) The central enzyme of the replication complex is the RNA-dependent RNA polymerase (RdRp) synthesising all viral RNA, whilst other enzymes contribute to initiation of replication, unwinding of the RNA, proofreading and sustaining the RdRp process. (5) Translated structural proteins translocate into the endoplasmatic reticulum (ER) and transit through the ER-to-Golgi Intermediate Compartment (ERGIC) to the Golgi for glycosylation and progression into secretory vesicles. Genomic viral RNA is shuttled into the cytoplasm and incorporated to (6) form the final virion that (7) is released from the infected cell through exocytosis. Right: Depiction of selected innate immune responses towards SARS-CoV-2 infection, combined with viral targets that subvert the human immune response. Image created with Biorender.com.*

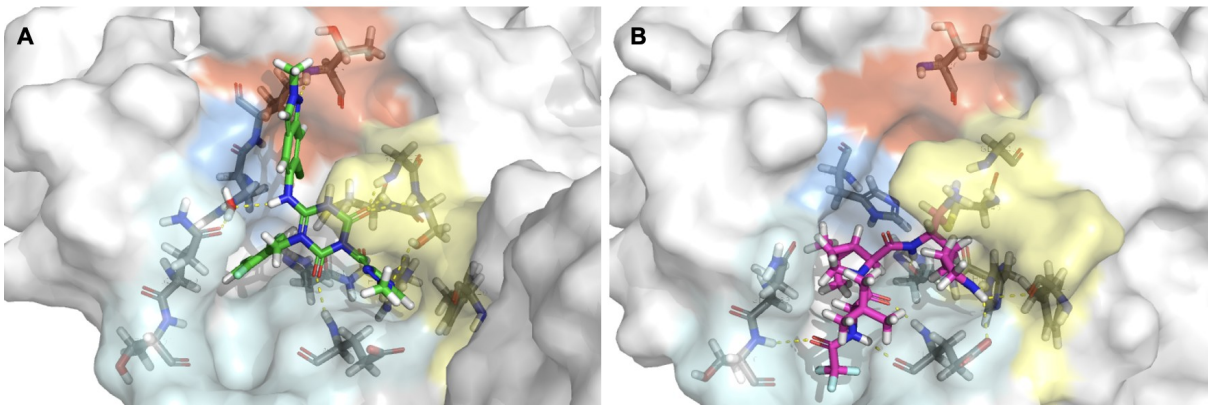


Figure 2. Crystallographic structure of SARS-CoV-2 main protease showing the active binding site with (A) Ensitrevir (PDB: 7VU6) and (B) Nirmatrelvir (PDB:7RFS). The colour shows the different binding pockets: P1' (orange), P1 (yellow), P2 (blue), and P3-5 (cyan).

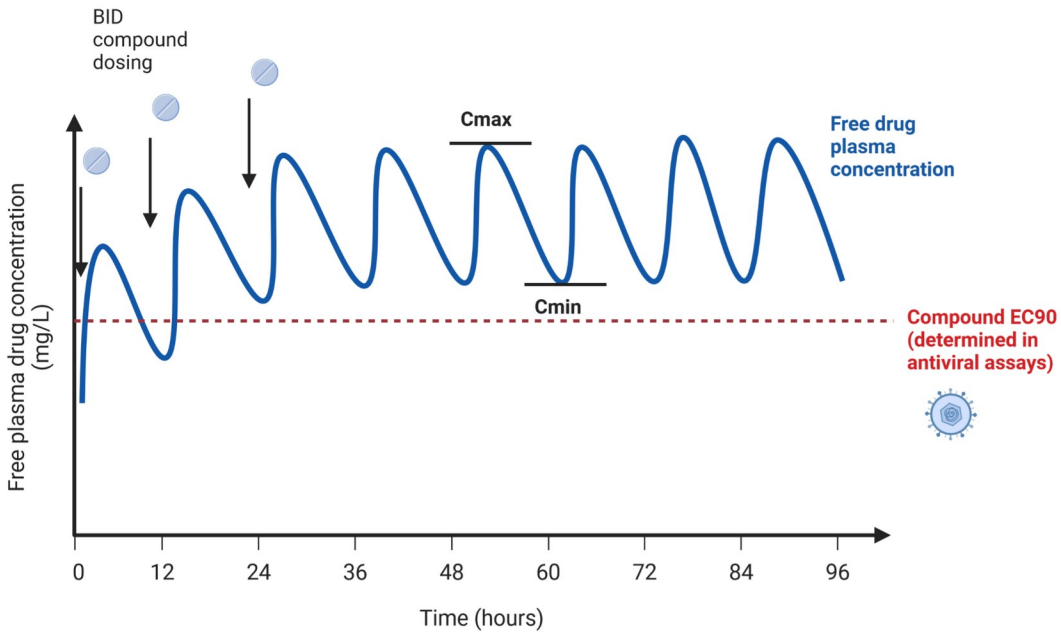


Figure 3: Schematic showing the pharmacokinetic profile of a hypothetical antiviral compound administered twice daily (BID). *The free (unbound) drug plasma concentration (blue) in mg/L is depicted, including the maximum drug concentration (Cmax) and minimum drug concentration (Cmin) at steady state. The 90% effective concentration (EC90), the concentration at which 90% inhibition of viral replication is observed in cellular antiviral assays is corrected for plasma protein binding. Image created with Biorender.com.*

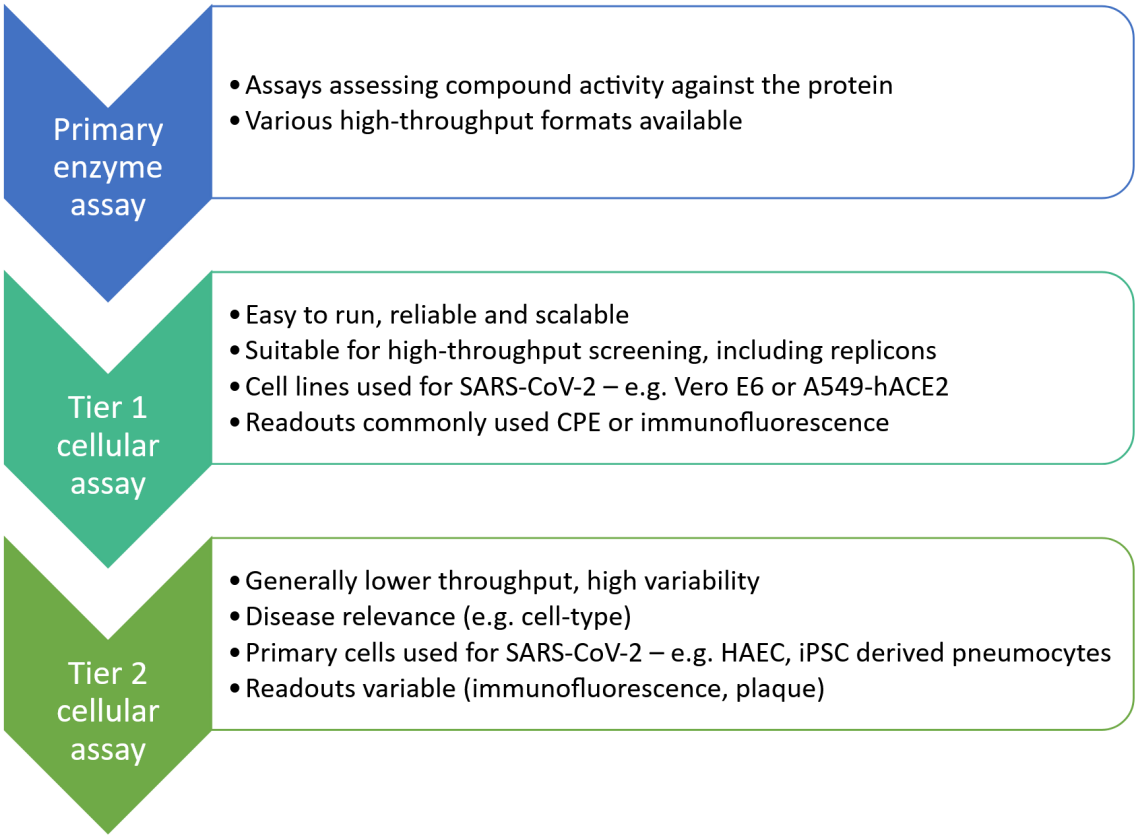
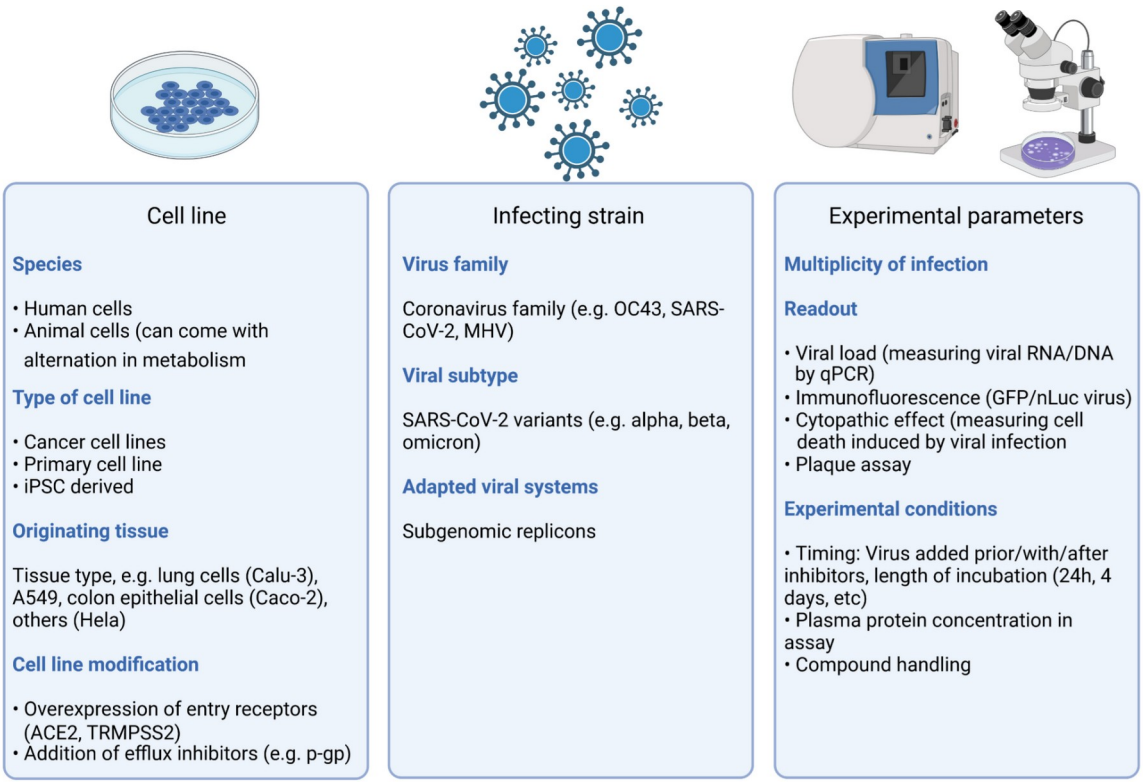


Figure 4: A cascade approach is typically employed for antiviral screening, where a high-throughput "Tier 1" assay is used routinely to drive medicinal chemistry, and a lower throughput "Tier 2" assay is used to predict human dose.



Textbox 1: Common variables in cellular antiviral assays that can impact the final readout. *Image created with Biorender.com.*