Understanding and Targeting Coronaviruses: exploring advanced cell culture models and host-directed antiviral strategies

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Chapter 6

Summary and General Discussion

Introduction

December 2019 marked the beginning of an unprecedented pandemic, with an enormous impact on health, society and economy worldwide. During the next four years, intensive research efforts have enhanced our understanding of many aspects of SARS-CoV-2 replication, pathogenicity and epidemiology, contributing to the rapid development of effective vaccines and antiviral drugs. Undoubtedly, decades of prior research on coronaviruses helped to understand and combat SARS-CoV-2 at such an unprecedented speed. Still, there are many aspects of SARS-CoV-2 and its associated disease COVID-19 that are not fully understood. Many healthy individuals will experience asymptomatic infections (1-3). If symptoms occur, they are primarily of a respiratory nature and can range from mild to severe, resulting in life-threatening pneumonia in a fraction of patients (4). However, the virus can also cause damage to other organs, like the cardiovascular, gastrointestinal, or nervous system, rendering COVID-19 a multisystemic disease. COVID-19 is unique in that it can cause acute olfactory dysfunction (anosmia), a loss of smell or taste that occurs more frequently than reported for other viruses, with the underlying mechanism remaining unclear (5, 6). Another phenomenon that is not fully understood, is post-COVID syndrome (also known as “long COVID”), a term used to describe a plethora of possibly debilitating symptoms that persist or arise after the acute infection, and can last for months or years. The development of post-COVID syndrome is associated with all disease severity, ages or vaccination status (7-10). Although the rapid development of vaccines and antiviral therapies was successful and helped to curb the pandemic, SARS-CoV-2 has now become endemic in the human population, with new variants continuously evolving (11) and causing periodic spikes in infection rates (12). Although the World Health Organization has declared the pandemic no longer a public health emergency, the evolution of SARS-CoV-2 and the emergence of new variants with possible changes in virulence continues to be monitored (12, 13). Omicron descendants, which are now, in early 2024, the dominant circulating strains, have increased transmission fitness (14, 15), changed their cell entry routes compared to early pandemic variants (16), and also cause olfactory dysfunction less frequently (17). New variants also show some level of evasion to natural or vaccine-induced immunity against earlier variants (16, 18, 19). In response, vaccines were updated (20). Continuous monitoring of SARS-CoV-2 evolution, as well as research and surveillance to detect zoonotic transmission of novel coronaviruses from their abundant animal reservoirs, will be crucial to detect new threats early on. While vaccines are efficient in curbing an outbreak of a known virus or new virus variants, by protecting a naïve population or reducing reinfections, their development takes time, and the demand for safe and effective antiviral drugs remains, for prophylaxis or treatment of early patients. Particularly broad-

spectrum antivirals would enable a faster response to combat (re-) emerging viruses, especially in an outbreak situation. Ideally, they would be stockpiled and made available quickly in the early phase of an outbreak. When vaccines are not yet available, such drugs could also be used prophylactically pre- or post-exposure. Furthermore, with COVID-19, patients often present with serious symptoms rather late, so there is not only a need for drugs that directly inhibit virus replication, but also for therapeutics that target the (pathogenic) host responses to infection, as well as treat post-COVID syndrome. One major challenge of all antiviral therapeutic approaches is the translation of drug efficacy and safety in preclinical models into clinical development. The use of advanced human cell culture models, that are more biologically relevant than immortalized cells and recapitulate the human tissue complexity, can help overcome this limitation.

The research projects described in this thesis, aimed to contribute to combatting SARS-CoV-2, and to increase our preparedness for the next emerging coronavirus. Besides screening for antiviral compounds, SARS-CoV-2 infection was characterized in an advanced infection model of human primary airway epithelial cells cultured at the air-liquid interface (HAE-ALI), aiming to better understand infection biology, epithelial host responses and the effect of antiviral drugs. Furthermore, SARS-CoV-2 and other coronavirus infections of HAE-ALI cultures revealed differences in host responses to high- and low-pathogenic coronaviruses.

Understanding coronaviruses through studying infection of advanced cell culture models (Chapters 2 and 3)

At the start of the SARS-CoV-2 pandemic, many researchers rushed to develop cell culture models in order to culture the virus and study its replication. Initially, the Vero E6 cell line was often used for studying SARS-CoV-2 and antiviral drug testing, but later the use of these cells was found to have some specific drawbacks. Besides the fact that they are African green monkey kidney cells and not representative of the human lung epithelium, they do not elicit an interferon immune response (21) and express high levels of an efflux transporter protein, which leads to increased cellular export of molecules like antiviral compounds, thus potentially obscuring efficacious compounds (22). The latter can be avoided by adding efflux inhibitors to the cell culture medium. Another disadvantage of using Vero E6 cells is the rapid acquisition of adaptive changes in the S protein of SARS-CoV-2. The SARS-CoV-2 S protein contains a furin cleavage site between the S1 and S2 domains, which plays a role in the high infection efficiency of SARS-CoV-2 (23). Upon passaging of (early pandemic variants of) SARS-CoV-2 in Vero E6 cells, several research groups reported that SARS-CoV-2 lost the furin cleavage site, resulting in drastic phenotypic changes (24, 25)

that improved viral fitness in Vero E6 cells, but reduced pathogenic properties *in vivo* (26). This is only one example that emphasizes that the choice of the right cell culture system for propagating and studying SARS-CoV-2 is crucial (24). Cell lines and organoids representing the liver, intestinal system, heart, brain, or kidney, have also been used (27-29), which might be relevant to study the implications of SARS-CoV-2 infections outside the respiratory tract. As the respiratory system is the entry point and primary target organ for SARS-CoV-2, the availability of *in vitro* infection models that represent the lung epithelium was crucial. Most frequently, conventional cell culture was used, with immortalized lung cell lines, like cancer-derived Calu-3 cell lines, immortalized human bronchial epithelial cells (30), or cell lines that are non-permissive but were modified to express higher levels of ACE2, like A549 or H1299 lung cancer-derived cells (31). More advanced cell culture infection models like human primary airway epithelial cells, cultured at the air-liquid interface (HAE-ALI), and organoids were also developed. Additionally, there are advances in the development and use of precision-cut lung slices or lung-on-a-chip models (32) that add further relevant infection models to the SARS-CoV-2 toolbox. The use of such advanced cell culture models has clear ethical and economic advantages over the use of animal models, and working with them is also less labour intensive and faster (33). At the same time, advanced models that use primary human epithelial cells better recapitulate the lung epithelium and the human tissue complexity (34), as opposed to monocellular laboratory-adapted immortalized or tumour cell lines.

Chapter 2 describes the characterization and optimization of SARS-CoV-2 infection in in-house produced ALI cultures of well-differentiated HAE cells. Using donor cells isolated from different anatomical locations, we found that ALI cultures of bronchial cells displayed increased SARS-CoV-2 infection compared to cultures of tracheal origin. The trachea and bronchi are at the beginning of the lower respiratory tract and contain similar cell types, mainly ciliated, goblet, club, and basal cells (35). Others have also found differences in infection based on the location in the respiratory system. Human nasal epithelial cells and upper airway cells *in vitro* were reported to be more permissive to infection than lower airway cells (36). High levels of virus replication in the upper airway could be linked to transmission efficiency, while high infection rates in the distal airway region of the alveoli is associated with severe disease symptoms and lung tissue damage, which was shown in non-human primate models as well as in deceased patients (37, 38). Single-cell sequencing data also revealed that a hyperinflammatory phenotype was enhanced in the bronchi compared to the nasopharynx (39). The difference in susceptibility between airway regions can be explained by the presence of susceptible cells containing the virus entry receptor and other co-factors. Ciliated cells, but also goblet or club cells are the main target cells of SARS-CoV-2 (40-43), as confirmed by our studies described in **Chapter 2**. Recently, a study analyzing

lung tissue from deceased patients with acute infection also confirmed ciliated cells as the main target cell type in the bronchial epithelium (38). Accordingly, our bronchial epithelial cell cultures, in which we observed higher viral infection, contained more ciliated cells than the cultures using cells derived from the trachea. A longer culture time also changed the cellular composition towards the presence of more susceptible cells and our transcriptome analysis revealed an increase in TMPRSS2 and CTSL expression, both host proteases facilitating virus entry into the cell, and expressed on ciliated cells (44). They are also both targets of antiviral drugs currently investigated for SARS-CoV-2 (**Table 1**). Besides the influence of the presence of ciliated cells (and virus entry factors) on viral infection, our study indicates a complex interplay of factors, in which the presence of goblet cells plays a role, as treatment with a γ-secretase inhibitor (DAPT), which shifts the epithelial differentiation entirely toward ciliated cells, did not increase virus replication compared to untreated cells. IL-13 treatment skewed differentiation towards more goblet cells, but with ciliated cells still present, resulting in (slightly) higher levels of infection compared to untreated cultures. Despite ciliated cells being the main target, there might be other factors in play, like the presence of the mucus that is secreted by goblet cells (45). Contrary to our results, one study showed a reduction in virus replication after IL-13 treatment (46), but in that study cell cultures were only treated for 48 hours, while our cultures were treated for two weeks to achieve differences in the cellular composition of the epithelium. Another study showed the upregulation of TMPRSS2 and downregulation of ACE2 expression by IL-13, however, this study focused on a different airway location, i.e. the nasal epithelium of children (47). Furthermore, that study and others also showed that ACE2 is an infection-mediated interferon-upregulated gene, which highlights the impact of virus infection itself on the expression of pro-viral factors like ACE2 and susceptibility of the epithelium (39). Allergic asthma, a disease associated with IL-13-induced changes in epithelial cell composition, was suggested to leave patients more vulnerable to a severe COVID-19 outcome, although reports are not always concordant (48, 49). Also chronic obstructive pulmonary disease (COPD) induces altered epithelial cellular composition, and was found to be a risk factor for severe COVID-19 (50). Our findings, that changes in epithelium cell composition (especially with ciliated and goblet cells of the mucociliary system) can impact the susceptibility to SARS-CoV-2, therefore could have implications for the disease outcome in patients with chronic lung diseases. Knowledge about the susceptibility of cells and the kinetics of virus replication and spread is important to understand virus transmission, pathogenesis, and evolution. A recent study showed that the SARS-CoV-2 variant Omicron BA.5, compared to its predecessors Omicron BA.1 and BA.2, more efficiently enters human lung cells and replicates better in the upper and lower respiratory tract of animal models (51). This indicates that it is not certain that continued subvariant evolution will only lead

to viruses that are less pathogenic, and that studying the replication kinetics and properties of new variants in advanced infection models remains crucial, to monitor risks.

To decipher the factors that determine replication kinetics and virulence, comparative studies (in advanced infection models) of different coronaviruses can provide valuable information. SARS-CoV and MERS-CoV are both highly pathogenic, but they use different cell-entry factors (52, 53), which can affect host cell tropism. A common cold coronavirus (NL63), causing only mild respiratory symptoms, utilizes the same ACE2 receptor (54) as the highly pathogenic SARS-CoV and SARS-CoV-2. This demonstrates that the determinants of pathogenicity are more complex than receptor use/cell tropism alone. Therefore, comparisons of host cell responses to high- and low-pathogenic coronaviruses in relevant infection models can help to understand the host and viral factors that truly play a role in pathogenesis.

Chapter 3 describes the differences in the host transcriptional response of HAE-ALI cultures to various coronaviruses. Although SARS-CoV-2 has been intensively studied since the start of the pandemic, only a limited number of comparative studies was done to learn from the differences and similarities between this pandemic virus and other human coronaviruses (55-60). One study utilized nasal epithelial cells cultured at the ALI and focussed on the infection kinetics and cell tropism of pathogenic and common cold coronaviruses, but did not investigate host responses (55). Some studies identified transcriptome changes induced by infection with pathogenic coronaviruses, but did not include common cold viruses (56, 57, 59), or combined already available datasets from different studies (57, 60). One of these studies employed a meta-analysis of available datasets for SARS-CoV and MERS-CoV from different studies (that used different cell culture models) and utilized the Calu-3 lung cancer cell line to compare differentially expressed genes in SARS-CoV, MERS-CoV and SARS-CoV-2 infections, but did not find major differences (57). Based on their analyses of common dysregulated pathways, they also performed a screen for potential drugs. In another study, analysis of the global transcriptomes of the cell lines Calu-3 and A549hACE2, infected with SARS-CoV-2, showed varying responses, underlining the impact of the choice of cell culture model on experimental results (61). Another study employed a biologically more relevant cell line of primary human lung epithelial cells and found differences in the responses to MERS-CoV and SARS-CoV-2 infection, mainly concerning immune-response-related genes. The study also reported an increased number of differentially expressed genes in MERS-CoV-infected cells compared to cells infected with SARS-CoV-2 (56).

In our study, we directly compared different high- and low-pathogenic coronaviruses side-by-side in the same advanced infection model, as the HAE-ALI cultures are susceptible to all human coronaviruses (**Chapter 3**). Our study corroborated the previously reported suppression or delay of an interferon response in cells infected with pathogenic

coronaviruses (61-63), but not with common cold coronaviruses (64), which highlights the relevance of the HAE-ALI cultures as a model for the *in vivo* situation. Interferon lambda (IFN-λ) was significantly higher expressed in cultures infected with HCoV-229E and HCoV-OC43, compared to highly pathogenic coronaviruses. Mucosal epithelial cells produce predominantly IFN-λ, which plays a crucial role in the antiviral defence against infections (65). IFN-λ treatment of HAE-ALI infected with SARS-CoV-2 led to a reduction in virus replication, confirming that suppression of interferon responses by pathogenic coronaviruses like SARS-CoV-2 favours their replication. The observation of decreased IFN-λ in critically-ill COVID-19 patients, and beneficial effects of treatment with pegylated IFN-λ in a mouse model (66) and in clinical trials (67, 68), supports this observation (69). We further identified differences in the transcriptional host response to infection with SARS-CoV-2 compared to the other coronaviruses SARS-CoV, MERS-CoV and HCoV-229E. Specifically, for SARS-CoV-2, we observed the down-regulation of a set of immediate early response genes related to the JNK/AP-1 pathway and NR4A1 expression. The results from our experimental infections were further supported by analysing available datasets from experimental and clinical studies (70-74). The AP-1 transcription factor, for example, regulates a range of cellular processes associated with apoptosis or inflammatory responses and was previously reported to be activated by SARS-CoV and HCoV-229E (75, 76). The role of NR4A1 in coronavirus biology has remained mostly unexplored so far. This protein is a master regulator of the stress response, is involved in regulating apoptosis and inflammation, and is associated with the immediate early response genes (77, 78). Suppression of the activity of this protein and associated pathways by SARS-CoV-2 may benefit the virus by evading innate immunity or other host responses, and may prevent the host cells from keeping early replication in check. The complexity of the JNK/AP-1 pathway, the exact implications of its up- or downregulation and its role in viral infection require further in-depth studies. For example, it remains to be investigated if the observed downregulation of the transcription factors is directly mediated by SARS-CoV-2 infection, and, if yes, which viral protein(s) is responsible. Proteomics studies could be used to validate our transcriptomics results and provide more information on how the observed changes in the transcriptome translate into changes in protein expression or modulation of pathways. Although we could confirm that the results from our *in vitro* (transcriptomics) studies were in line with clinical datasets, the question remains if and how these transcriptional changes play a role in pathogenesis *in vivo*. Despite the fact that we did not elucidate the exact role of NR4A1 in coronavirus replication, it appears an interesting target for follow-up studies and possibly for therapeutic strategies, as we found that an NR4A1 antagonist reduced replication of SARS-CoV-2 and MERS-CoV. This emphasizes the significance of studying

coronavirus biology to identify new potential host targets for the advancement of drug development.

Targeting coronaviruses through host-directed antiviral strategies (Chapters 4 and 5)

With the SARS-CoV-2 pandemic, there was a surge in antiviral drug discovery efforts, as scientists and clinicians raced to combat the virus and its associated disease, utilizing repurposing strategies as well as the development of new drugs. As mentioned in **Chapter 1**, currently there are only four drug therapies approved to treat COVID-19 in Europe (additionally, molnupiravir has still EUA by the FDA). Two of these are the direct-acting inhibitors of virus replication ritonavir-boosted nirmatrelvir (Paxlovid) and remdesivir (Veklury) (79, 80). The other two approved therapies concern the host-directed tocilizumab (IL-6 receptor antagonist) and baricitinib (JAK kinase inhibitor), which modulate the innate immune response and suppress a hyperinflammatory response (81-83). Approved anti-SARS-CoV-2 monoclonal antibodies, that target the S protein, are conditionally recommended or not authorized, as the dominant Omicron subvariants are not expected to be susceptible (84-86). Furthermore, other immunomodulatory drugs, like the corticosteroid dexamethasone, were found to reduce mortality by inhibiting hyperinflammation in critically-ill patients (87, 88). Therefore, host-directed strategies have been successful in tackling COVID-19 pathogenesis. So far, there are no host-directed antivirals (HDAs) that inhibit SARS-CoV-2 replication, although efforts to identify them are ongoing, as described below. HDAs that target host factors that are important for the replication of various (corona)viruses are especially interesting antiviral drug candidates as they have the potential to have broad-spectrum antiviral activity and thus would be beneficial to combat a newly emerging virus in an outbreak situation. **Chapters 4 and 5** describe two different classes of host-directed antiviral molecules, which both efficiently reduce the replication of SARS-CoV-2 and other coronaviruses.

Chapter 4 describes propranolol, a drug that is approved for various medical conditions, including cardiovascular problems and hemangioma, which is a benign tumor that develops through dysregulated blood vessel formation (89, 90). Propranolol is a mix of two stereoisomers, R- and S-Propranolol. R-propranolol was reported to lack the beta-blocker activity of its S-stereoisomer and therefore might induce fewer side effects in patients (91), which is why we investigated R-propranolol. We initially evaluated R-propranolol for its ability to inhibit proangiogenic (transcription) factors, reported in the context of hemangioma research (91), because the list of pathologies described for COVID-19 includes

vascular disease, associated with endothelial dysfunction and pathological angiogenesis in the lungs of patients (92). In a recent study, increased expression of biomarkers involved in angiogenesis was observed in COVID-19 patients, compared to influenza patients or control groups (93). Factors like vascular endothelial growth factor (VEGF), VEGF receptor 1, matrix metalloproteinase 2 (MMP-2), hypoxia-inducible factor 1 α (HIF-1α), angiopoietin 2 (Ang-2), TGF-β, angiopoietin-like proteins, and others are mentioned as biomarkers in COVID-19. Another proangiogenic factor, angiopoietin like 4 (ANGPLT4), is described in the literature as a key player in angiogenesis, often with a detrimental role in respiratory virus infections like Influenza (94). Very recently, increased plasma concentrations of ANGPTL4 in COVID-19 patients were correlated with an increased rate of ARDS and mortality (95). In our project, using Hulec-5a human lung endothelial cells, we confirmed increased expression of *angptl4*, when we mimicked the endothelium-epithelium environment during infection. We did so by treatment of Hulec-5a with conditioned medium from Calu-3 lung epithelial cells, as Hulec-5a are not susceptible to SARS-CoV-2 infection (96). Another study utilized a more advanced setup of alveolar epithelial cells at the ALI with endothelial cells co-cultured on the other side of the membrane (96), a design that represents the epithelial-endothelial crosstalk even better. Treatment of Hulec-5a cells, which were chemically induced to express *angptl4*, with R-propranolol led to a reduction of *angptl4* expression compared to untreated cells, confirming the anti-angiogenic properties of R-propranolol (91). Besides the suppression of the proangiogenic factor, we also discovered an antiviral effect of R-Propranolol, which we observed also in experiments with SARS-CoV, MERS-CoV, and the SARS-CoV-2 variants delta and omicron. Prior to this project, only some clinical evidence of a potential antiviral effect of propranolol had been published, suggesting activity against herpes simplex or influenza virus (97). Confirmation of a potential broad-spectrum activity of R-propranolol would need further evaluation in clinical studies. Another study, which is available as preprint, observed antiviral activity of propranolol against SARS-CoV-2, mouse hepatitis virus, Dengue virus, and Zika virus, and suggested that the compound affects replication complex formation through an effect on phospholipid synthesis (98). Propranolol also has an effect on various other host factors and signalling pathways, including inhibition of the RAS/RAF/ERK and AKT pathways (99, 100). Inhibition of factors involved in these signalling pathways was also shown to affect SARS-CoV-2 replication (101). Propranolol was also suggested to have immunomodulatory effects, although through its effect on the sympathetic nerve system by beta-blocker activity (102). Due to R-propranolol's (potential) broad-spectrum antiviral activity it is likely that propranolol exerts its antiviral activity through targeting host factors that play a role in virus infection, but more in-depth research is required to elucidate the underlying mechanisms, and to establish if there are multiple angles of activity and if this depends on the virus. RNA sequencing

analysis of host transcriptional changes could be a starting point for further investigation. The dual-activity of R-propranolol to act as potent (broad-spectrum) antiviral and possibly limit proangiogenic responses, which could play a role in reducing lung pathology in COVID-19 patients or those suffering from other serious respiratory virus infections, makes it an interesting candidate to investigate further and elucidate the mode of actions. In an ideal situation, certain risk groups of patients could be tested for biomarkers, like those for pathogenic angiogenesis, to predict the severity of the disease, allowing the early initiation of appropriate (preventative) treatment. The suitability of the use of such biomarkers is currently being assessed (103) and drugs to prevent endothelial dysfunction through anti-angiogenic action, like the VEGF inhibitor bevacizumab, are being evaluated for the treatment of COVID-19 lung pathologies in clinical trials (104). In our project, it would have contributed additional supportive information to also evaluate the effect of R-propranolol on VEGF for comparison with bevacizumab. Furthermore, future studies will need to address the effect of R-propranolol in more advanced models (organoids or *in vivo*), to recapitulate the pathogenesis of angiogenesis and see if the compound has a therapeutic effect on exacerbated endothelial dysfunction. Finally, existing pharmacological knowledge of propranolol and its two stereoisomers could aid in the development of R-propranolol as a repurposed antiviral drug.

Chapter 5 describes another class of host-directed antiviral compounds: glucosidase inhibitors. SARS-CoV-2, like other viruses, uses the host machinery for post-translational modifications of their proteins, including glycan processing in the endoplasmic reticulum (ER), which is involved in proper protein folding (105, 106). The surface of the most prominent SARS-CoV-2 structural protein, the Spike (S) protein, is covered with N-glycans, which are important for protein stability and function (107, 108). Using small molecule drugs, which inhibit α-glucosidase enzymes I and II in the ER (ER α-Glu I/II), prevents the production of viral glycoproteins and blocks replication of viruses that rely on the ER-protein quality control (105). Decades of research on iminosugars, a class of glucosidase inhibitors, reported the inhibition of a number of viruses (109-112), including most recently SARS-CoV-2 (113-115), however none have proceeded beyond phase II clinical trials as antivirals (116, 117). We screened a collection of iminosugars and another class of glucosidase inhibitors, cyclitols. We identified 1,6-*epi*-cyclophellitol cyclosulfate as the most potent and selective inhibitor of ER a-Glu II (118), and with the highest antiviral activity against SARS-CoV-2. Selective inhibition of ER a-Glu II by 1,6-*epi*-cyclophellitol cyclosulfate prevents the production of SARS-CoV-2 infectious virus particles through blocking of S protein N-glycosylation, and not through blocking of viral entry into the host cell or replication of the viral genome. Compared to the selective activity of 1,6-*epi*-cyclophellitol cyclosulfate against ER a-Glu II (with the lysosomal retaining a-glucosidase, GAA reported as the only off-

target effect) (118), iminosugars also target ER-α Glu I and other human glycoprocessing enzymes (119, 120), possibly leading to adverse effects. For example, for Hepatitis A virus infection, treatment with iminosugars was suggested to enhance virus entry, through inhibition of β-glucosidases which interferes with ganglioside degradation (121). Notably, it would add to our study to have an enzyme activity assay to assess the specific inhibition of ER α-Glu I by the compounds and confirm ER α-Glu II-selectivity of 1,6-*epi*-cyclophellitol cyclosulfate. Furthermore, as reported in a phase II clinical trial with an iminosugar compound (122), toxicity has to be evaluated in long term treatment. In that study, there was no concern about short term or emergency treatment, as the concentrations that are necessary for antiviral activity, appear to affect viral glycoproteins more than cellular glycoproteins. Also in our study, we did not observe toxicity, even at high concentrations of compounds used, that is, at least in short term treatment *in vitro*. Furthermore, we observe a potency limitation for 1,6-epi-cyclophellitol cyclosulfate, i.e. the antiviral activity reaches a plateau when higher concentrations of compound are used. The maximum antiviral effect is already reached at 0.5 µM, a concentration at which also an almost full inhibition of ER α-glucosidase II activity was observed. This could be a result of proteins escaping the glucosidase machinery in the ER through an endo-mannosidase dependent mechanism that acts in the Golgi (123, 124). Mechanistically, this escape route may account for the inability of glucosidase inhibitors to completely inhibit viral glycoprotein production. Further studies should determine whether other enzymes can complement the inhibition of ER α-glucosidase II. In one study combination of glucosidase I/II inhibitor and endo-mannosidase inhibitor indeed reduced virus replication (124). Glucosidase inhibitors in general do not exert activity in the initial infection, but are effective at reducing spread and therefore have potential as drug candidates that can be used later in infection, or used in combination therapy with other antivirals. Their broad-spectrum activity against various viruses make them interesting candidates in HDA strategies that could be employed during an outbreak with a new virus when urgent treatment options are needed. The selective blocking of ER α-Glu II, linked to potent antiviral activity, presents a new strategy in the search for effective antiviral compounds targeting SARS-CoV-2 and other viruses that rely on ER-protein quality control for replication.

Importance of advanced cell culture models in drug research

Animal-free models are increasingly implemented in (bio)medical research, driven by ethical concerns, advancements in innovations and increased funding support. To optimize the evaluation of drug candidates and shorten the way from pre-clinical research to clinical

trials, the use of the right *in vitro* cell culture model is crucial. Besides ethical advantages, animal-free drug-testing models cost less and are less labour-intensive, thus saving time and manpower. Compared to the use of animal models, studying new drugs in a system that closely mimics the human tissue complexity and physiology might even be more reliable in predicting human responses. Organoids, organ-on-chip models, or ALI cultures derived from primary human cells better mimic the characteristics of infections *in vivo*, host responses, or drug effects compared to some conventional cell culture models, based on the use of immortalized or cancer-derived cell lines and lacking tissue complexity (34, 70, 125). Currently, tests in animal models are still required before moving therapies or vaccinations into clinical trials with humans, but innovation in animal-free science moves fast. Only in 1998, scientists were able to isolate and culture human embryonic stem cells for the first time, and later, in 2007, induced pluripotent stem cell research followed, which really started 3D organoid research (126). Since then 3D models using human cells have become a standard tool in many fields like infectious disease modelling and drug discovery, to evaluate the efficacy and toxicity of a drug (127). One study reported on an extensive high-throughput drug screen in lung and colonic organoids (128). Recently, another study evaluated the efficacy and toxicity of reference antiviral drugs, which either succeeded or failed in late stage clinical trials, in human small intestine organoids and showed the reliability of the organoid model (129). In 2010, the first lung-on-chip model was described (130). Organ-on-chip models include microfluidics, allowing for a continuous exchange of used and fresh culture medium and nutrients, or introducing mechanical stimulation, like stretching in the case of simulation of the lung epithelium (131). A lung-on-chip model constituting epithelium and endothelium could successfully be infected by SARS-CoV-2 and used for treatment with an IL-6 receptor antagonist (132). Most frequently used in SARS-CoV-2 research and preclinical drug discovery were primary human airway epithelial cells that are differentiated at the air-liquid interface (HAE-ALI). These cultures are more accessible and less elaborate than organ-on-chip models, while recapitulating the pseudostratified epithelium of the human lung (133). Technological advances, like bioprinting, will automate the generation of such cultures in a reproducible and high-throughput fashion (134). Like organ-on-chip models, HAE-ALI cultures also provide the option of adding endothelial cells or immune cells, thus moving closer to simulating human tissue complexity (96, 135). Other advantages of using advanced lung cell culture models are that the number of experimental animals can be reduced, and that more relevant research results can be obtained with better reliability in predicting human responses. One study reviewed molecular screens of host factors that are important for coronavirus infection and reported high variability in the findings when different cell lines were used (136). Especially, results obtained with the often used Vero E6 cells do not always translate

to the situation in the lung epithelium *in vivo*. Chloroquine, ivermectin, or favipiravir are examples of repurposed drugs that showed promising results when tested on Vero cells (137) but had no benefits for patients suffering from COVID-19 (138-140). Later it was shown that these drugs do not protect human lung cells from SARS-CoV-2 infection (138, 141, 142). The same scenario occurred for the tyrosine kinase inhibitor imatinib (143). This illustrates the importance of using appropriate cell culture models for antiviral drug screening, or additional evaluation in preferably primary human cells, already at the stage of pre-clinical research, to decrease the resources and development time and also shorten the time until failure (**Figure 1**). Using primary human cell models therefore bridges the gap between *in vitro* and *in vivo* studies; especially now that the FDA Modernization Act 2.0 permits incorporation of results from pre-clinical *in vitro* studies and allows alternatives to animal testing (144, 145). These regulatory changes, alongside the development of advanced human cell culture models *in vitro*, are changing the landscape of drug development. Although the final evaluation of drug candidates still needs to be done in human clinical trials, the use of advanced cell culture models can shorten the way from pre-clinical to clinical development and increase the probability of success.

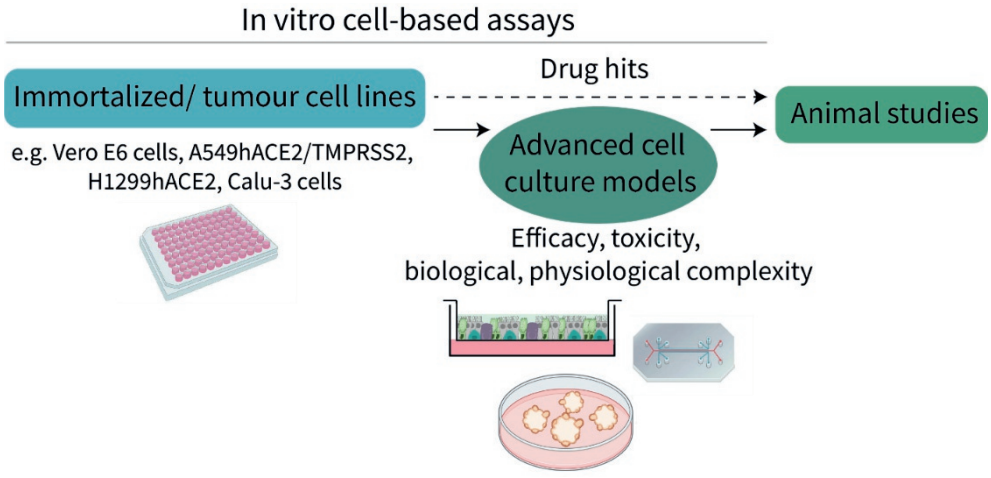

Figure 1: Advanced cell culture models for infection and drug testing, that recapitulate human tissue complexity and provide reliable predictive data on drug efficacy and toxicity have an important role in increasing efficiency in the drug development pipeline, by increasing the success rate of a drug to progress from the pre-clinical to the clinical phase. These models will also aid in reducing the number of animal experiments.

Current and future landscape in SARS-CoV-2 host-directed antiviral drug development

Many factors have to be taken into account in the context of antiviral drug development, both for the assessment of repurposed drugs and the development of new potential therapies. The whole drug development pipeline is time-consuming and cost-intensive (146). That is why the idea of repurposing already studied or approved drugs is appealing, especially in the context of a new outbreak, where time is crucial. The use of artificial intelligence (AI) will accelerate drug discovery, as it can scale up the target and hit identification process (147). Lead optimization efforts benefit from the information that AI-based models can predict, which can for example be pharmacokinetic (adsorption, distribution, metabolism, excretion) or toxicity properties (148). AI tools can further be used for the prediction of synergistic drug combinations. Of course, new technology also creates new challenges. The combination of new technologies like AI with human expertise is crucial, to assure input of good-quality, standardized data and confidence in interpreting results. In recent years, advances in AI made it possible for pharmaceutical companies and university research laboratories to collaborate and share their data about small-molecule drugs to enable more accurate predictive machine learning-based drug discovery models (149, 150). Through these collaborations, already available proprietary data, which would otherwise remain classified, could be shared by collecting it in a decentralized database.
For the treatment of SARS-CoV-2, target-based development of new antivirals has yielded effective drugs. The most successful SARS-CoV-2 antiviral to date, nirmatrelvir (Paxlovid), directly targets the viral main protease (80). The fast development of nirmatrelvir was made possible through pre-existing knowledge about the SARS-CoV main protease and inhibitors (drug candidates) against it (151). Thus, repurposing strategies in drug development should not only encompass approved drugs, but should also leverage scientific knowledge and existing antiviral compounds that did not (yet) reach advanced (clinical) stages in drug development. Although repurposing efforts for treating SARS-CoV-2 infections had limited success, there is still great potential in collecting knowledge about clinically relevant drugs, providing this knowledge preferably in open-access databases to make it available for future research. Meanwhile, other inhibitors that target the main protease of SARS-CoV-2 are being investigated, like masitinib (152) or ensitrelvir (marketed as Xocova), which received emergency approval in Japan at the end of 2022 (153). The drug showed very promising results in a phase II clinical trial, but is further evaluated in phase III clinical trials before authorization outside of Japan. Direct-acting drugs like these, with high specificity against a viral protein that is well conserved among coronaviruses, will likely retain their efficacy against new circulating variants and subvariants (154). However, persistent infections in

patients treated with direct-acting drugs can lead to resistance development, as was reported in various studies for the protease inhibitors (155, 156), as well as remdesivir (157, 158). Therefore, monitoring for new resistant variants is important, as well as the development of next generation protease inhibitors (155). Furthermore, combination therapy, the use of more than one drug, can be employed to reduce the risk of resistance development (159).

Given the systemic and long-term pathology that can be caused by SARS-CoV-2 infection, researchers are challenged with the task of developing drugs that not only inhibit virus replication and treat acute symptoms, but also protect from tissue damage and hyperinflammation. Clinicians are faced with the challenge to get the timing right when treating patients with DAAs to reduce viral load, or with anti-inflammatory drugs when the disease is more advanced (139). Treatment with protease inhibitors, for example, needs to start during the early stage of infection. Therapies to control pathological host responses and prevent disease progression in the lung or other organs, or alleviate long-term consequences of COVID-19 are at least equally important, as well as treatment to restore functionality of damaged organs or tissues. Combination therapy, besides having the potential to reduce virus resistance development and increase antiviral efficacy, could be used to target virus replication and pathological responses at the same time (159). For example, combination of remdesivir and baricitinib was found to improve recovery time in hospitalized patient compared to remdesivir mono-therapy (160). An ongoing study also investigates the use of a combination of a direct antiviral drug, the main protease inhibitor masitinib (152), and the immunomodulatory drug isoquercetin (161) (**Table 1**). Now, as we have moved past the SARS-CoV-2 pandemic being an acute threat to public health, research efforts are also switching to find therapies to treat long-term consequences of infection, like post-COVID syndrome, and lung pathologies, like fibrosis (162, 163). Researchers are aiming to bridge the gap between elucidating the molecular processes during infection and understanding clinical outcomes. Immune-modulatory drugs are crucial in the treatment of severe COVID-19, like the approved drugs tocilizumab or baricitinib, which are host-targeting and reduce the hyperinflammation that can follow SARS-CoV-2 infection (82, 83). Besides many pre-clinical studies reporting on potential candidates for host-targeting therapies, a number of these treatments are currently also evaluated in clinical trials. Following up on a review (164), a search of clinicaltrials.gov in December 2023 for interventional host-targeting therapies, yielded a list of potential drugs that either aim to inhibit virus replication or treat SARS-CoV-2 induced pathologies through targeting host factors (**Table 1**). SARS-CoV-2 neutralizing monoclonal antibody therapies, of which there are now eleven marketed and many more investigated in clinical trials (165), were not included in **Table 1**. The search was limited to active trials for interventional therapies, and

therefore the table also does not include trials that are not yet recruiting or are currently recruiting, like US trials for the protease inhibitor ensitrelvir. Notably, some of the inhibitors have the potential to act as broad-spectrum antivirals, like for example serine protease inhibitors that affect the cleavage of viral surface glycoproteins and inhibit virus entry (166). Also kinase inhibitors to treat the pathologies that follow virus infection, like hyperinflammation or lung fibrosis (163), have the potential to be applicable for a broader range of virus infections. The landscape of antiviral treatment of COVID-19 is rapidly changing, with hundreds of trials currently active or recruiting participants, and therefore **Table 1** merely provides a snapshot summary of the current situation and a look into the future of COVID-19 therapy development**.**

Table 1. Host-directed antivirals evaluated in clinical trials

Drug	**Target**	**Effect**	**Clinical trial**	**Clinical trial reference**
Meplazumab	CD147 receptor	Inhibition of virus entry (167)	Phase III	NCT05679479
SLV213	Cathepsin	Inhibition of virus entry (168)	Phase II	NCT04843787
Nafamostat mesylate	Transmembrane serine protease 2 (TMPRSS2)	Inhibition of virus entry (169)	Phase II and III	NCT04352400
Upamostat	Serine proteases	Inhibition of virus entry (166)	Phase II	NCT05954286
Plitidepsin	Host-translation cofactor eEF1A	Inhibition of virus replication (170)	Phase II	NCT05705167
TXA127, TRV027;	*Renin-Angiotensin-Aldosteron-System*	Reduction of fibrosis (171)	Phase II and III;	NCT04924660
Fostamatinib	*Spleen tyrosine kinase*	Reduction of thrombosis (172)	Phase II and III	NCT05593770
EB05	TLR4	Reduction of TLR-4 mediated IL-6 release / hyperinflammation	Phase II and III	NCT04401475
LAU-7b	Fatty acid metabolism	Inhibition of lipogenesis (173)	Phase II and III	NCT04417257

Imatinib	Abl tyrosine kinase	Inhibition of hyperinflammation (174)	Phase III	NCT04394416
Isoquercetin (in combination with masitinib)	Oxidation-Inflammation response	Inhibition of hyperinflammation (161)	Phase II	NCT04622865
Nintedanib	Tyrosine kinase	Inhibition of lung fibrosis (163)	Phase IV	NCT04619680

The website clinicaltrials.gov was searched for drug candidates that are currently in active clinical trials for the treatment of COVID-19 and are interventional and targeting host factors (December 2023). Drugs were listed only if there is a known or proposed mode of action.

Concluding remarks

Decades of coronavirus research and intense efforts to curb the SARS-CoV-2 outbreak have led us to understand many aspects of the viral replication cycle and pathogenicity. This thesis highlights some of my work, in collaboration with many others, in the context of anti-coronavirus drug research. The ultimate goal, of course, would be a broad-spectrum antiviral drug targeting all current highly-pathogenic coronaviruses (and future ones), or a universal vaccine, which seems to be an even more challenging task, given the rapid evolution of SARS-CoV-2 subvariants and their escape from previously developed immunity. However, with efficient development platforms in place, such as mRNA vaccine development platforms or portfolios of known antivirals ready for further development, the response time to a newly emerging disease can be reduced. Furthermore, the implementation of advanced cell culture models/animal-free models to validate drug candidates is an extra step that can increase the success rate from pre-clinical to clinical research. Continuous monitoring of SARS-CoV-2 and ongoing efforts in adapting vaccines and developing antivirals are crucial to combat new subvariants. The accumulated knowledge about coronavirus biology as well as public health infection prevention methods hopefully leaves us better prepared for any new epidemic or pandemic.

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