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

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Chapter 1

General Introduction
and
Outline of the Thesis

Introduction

Viruses are biological entities that can infect host organisms from all domains of life, from prokaryotic microorganisms, such as bacteria and archaea, to plants, animals, and humans. Their replication occurs intracellularly and is dependent on the infrastructure and metabolism of the host cell. Viruses have been evolving alongside (in other animal hosts) or with humans since the dawn of humanity. As ancient as viruses are, they are subject to rapid evolution, especially when we look at viruses with an RNA genome, which have high mutation rates and great genetic diversity (1). These viruses continuously adapt to changing environments through changes in their tropism, transmission, replication, pathogenicity, or immune evasion. Escape from neutralizing antibodies or antiviral drug treatment is also driven by their potential for rapid evolution, resulting in antibody or drug resistance of an adapted virus population. To combat viruses we need vaccine and antiviral drug strategies, and to develop such it is crucial to study and understand viruses in detail. Coronaviruses in particular, besides other respiratory virus groups, are endemic in the human population as common cold viruses, which are low pathogenic viruses that usually cause minor symptoms in the upper respiratory tract of healthy individuals. However, coronaviruses, like many other virus groups, also have a great zoonotic potential (2), which has led to three outbreaks in humans since the beginning of the 21st century. In the past two decades, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and most recently SARS-CoV-2, the causative agent of COVID-19, have crossed the species barrier. The zoonotic potential of coronaviruses, as well as the continuously evolving variants of SARS-CoV-2, underscore the risk of new pandemics. Enhancing our understanding of how a virus can cause a pandemic and studying antiviral approaches against a broad-spectrum of viruses can help to prepare us for future viral threats.

In this thesis, I will describe our efforts to add to this understanding by studying coronavirus infection and host responses to infection using an advanced human cell culture model. Moreover, I demonstrate how coronavirus replication in cell culture models can be inhibited by targeting the host cell with small-molecule compounds. Given the SARS-CoV-2 outbreak in late 2019, this pandemic virus was the main focus of my research project.

Coronaviruses

Coronaviruses (CoVs) belong to the subfamily *Orthocoronavirinae* (family *Coronaviridae)* of the order of *Nidovirales*, and are enveloped, positive-sense single-stranded RNA viruses that

infect mammalian and avian species (3, 4). *Orthocoronavirinae* are classified into four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus (4)*. *Gamma-* and *deltacoronaviruses* mainly infect birds (5). The *alpha-* and *betacoronavirus* genera comprise, besides others, the seven coronaviruses that infect humans and are known to date (**Figure 1**). The human alphacoronaviruses 229E and NL63 as well as the human betacoronaviruses HKU1 and OC43 circulate in the human population as common cold viruses, and in healthy individuals they only cause mild upper respiratory tract symptoms (6). However, the other three coronaviruses that can infect humans can cause severe clinical symptoms: SARS-CoV, MERS-CoV, and SARS-CoV-2; all three belong to the *Betacoronavirus* genus. Due to their zoonotic emergence in the past two decades, they have gained global public health attention. The first epidemic outbreak of a highly pathogenic coronavirus in the 21st century started in 2002 and was caused by SARS-CoV (7), which spread mostly in Southeast Asia, where it could be contained within a year. MERS-CoV, which was first identified in 2012 in Saudi Arabia (8), is still causing small outbreaks in the Middle East. Both viruses likely originated in bats, but transmission to humans is thought to have occurred through intermediate hosts, like civet cats for SARS-CoV and dromedary camels for MERS-CoV (9). Recent studies in the human population have shown that there is also transmission of MERS-CoV from dromedary camels to humans in Africa (10). Furthermore, numerous coronaviruses are now known to circulate in a range of bat species, highlighting the risk of new zoonotic outbreaks (9, 11). Most recently, at the end of 2019, SARS-CoV-2 caught the world off guard and caused a pandemic of unprecedented impact on health care systems, societies, and economies worldwide (12). In contrast to SARS-CoV and MERS-CoV, this new virus spread quickly across the world. SARS-CoV-2 was first reported in Wuhan, China, and is suspected to be of zoonotic origin, as closely related viruses were found in bats and pangolins (13, 14). SARS-CoV-2 is closely related to SARS-CoV, as they share 79.6% nucleotide identity in their genomic sequences (15) and belong to the same phylogenetic subgenus classification, *Sarbecovirus.*

Although the emergence of SARS-CoV and MERS-CoV had put coronaviruses on the map as potentially lethal agents for humans, and coronavirus research had spiked since then, still the world was not prepared for SARS-CoV-2, as there were no approved vaccines or antiviral drugs available. While it seemed the world was brought to a halt in order to stop the spread of the pandemic, health care personnel and scientists raced to understand the new virus and the disease it can cause and tried to identify or develop vaccines and drugs. Consequently, in 2020, coronavirus research output increased by a hundredfold (in the first pandemic year, in PubMed the number of publications on coronaviruses increased from about 800 in 2019 to 80,000 in 2020).

Over the past 4 years, SARS-CoV-2 has evolved and different variant strains have been circulating. Through variant classification, the World Health Organization (WHO) and the European Center for Disease Control (ECDC) keep track of so-called variants under monitoring (VUM), variants of interest (VOI) and variants of concern (VOC). In particular the latter have predominantly circulated across the world and are known as the Alpha, Beta, Gamma, Delta, and Omicron variants of SARS-CoV-2. Since March 2023, there have been no new SARS-CoV-2 variants reported that meet the VOC criteria, although monitoring shows that SARS-CoV-2 is undoubtedly still circulating in the human population. In May 2023 the WHO declared that COVID-19 no longer constitutes a public health emergency of international concern, but instead has become an established and ongoing health issue (16). Descendants of the Omicron variant continue to prevail and evolve into new subvariants. Most recently, the variant of interest (VOI) that is steadily spreading is JN.1, an Omicron BA.2.86-like lineage, which is slowly replacing the XBB descendant lineages (17). Although the WHO no longer considers the SARS-CoV-2 pandemic a public health emergency, the number of cases in the winter of 2023-2024 illustrates that this virus is here to stay and for now continues to require management and surveillance on a daily basis.

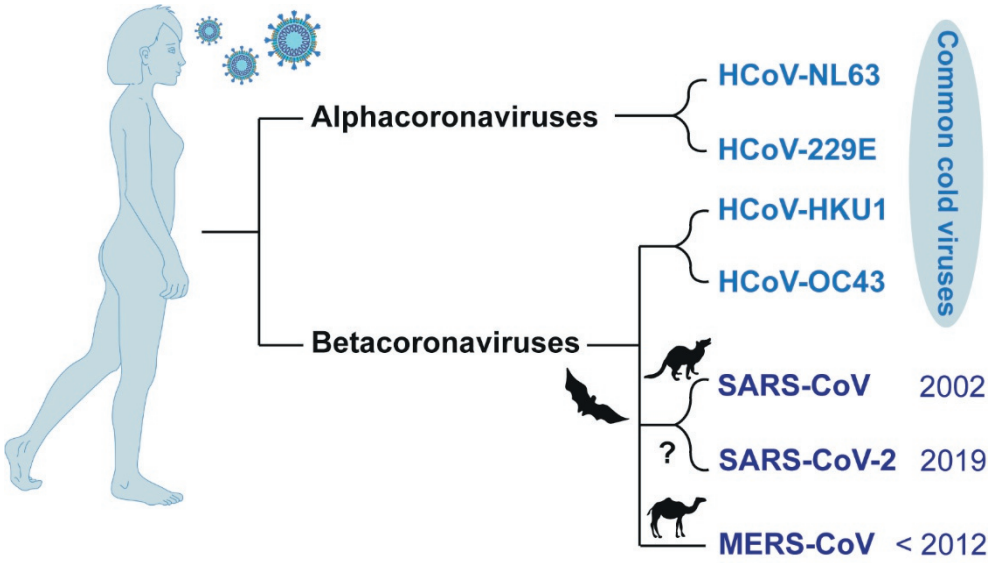

Figure 1: Seven coronaviruses are known to date that infect humans: The low pathogenic coronaviruses that cause the common cold are the alphacoronaviruses NL63 and 229E, and the betacoronaviruses HKU1 and OC43. The possibly highly pathogenic coronaviruses are all betacoronaviruses, comprising SARS-CoV and SARS-CoV-2 and MERS-CoV. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

Coronavirus replication

Coronaviruses are spherical virions with a diameter of around 100 nm (18). They are enveloped viruses with a lipid membrane containing three structural proteins: membrane (M), envelope (E), and spike (S) protein (19). The virion core (nucleocapsid) consists of a 30-kilobase long positive-sense single-stranded RNA molecule, containing a 5' cap structure and a 3' poly (A) tail, which is encapsidated by the nucleocapsid (N) protein. The S protein is present on the virion surface as a homotrimer and is the most prominent envelope protein, as it is studied in detail for its role in infectivity and host immunity. It orchestrates host cell attachment and virus entry, is the main target of neutralizing antibodies and undergoes significant changes during SARS-CoV-2 variant evolution (20, 21). The S proteins that protrude from the surface of coronaviruses are seen as a crown, or "*corona*" in Latin, when using an electron microscope, which was the basis for the name of the coronavirus family (22). The S protein consists of two subunits, S1, which is responsible for attachment, and S2, which enables the fusion of the viral and host cell membrane (23). The S1 subunit contains the receptor-binding domain (RBD), which is the point of contact for the host receptor as well as the main target of neutralizing antibodies (24). Via the RBD, SARS-CoV and SARS-CoV-2 bind predominantly to the angiotensin-converting enzyme 2 (ACE2) receptor (25, 26), while MERS-CoV binds to dipeptidyl peptidase 4 (DPP4) receptor (27). HCoV-NL63 also binds to ACE2 (28). In turn, the other common-cold coronaviruses again utilize different host receptors, with HCoV-229E binding to human aminopeptidase (hAPN), and OC43 and HKU1 to 9-*O*-acetylsialic acids (29, 30). For variants of SARS-CoV-2, like the delta variant, amino acid changes in the S1 subunit have been reported to enhance binding to ACE2 and increase replication and transmissibility (31). Essential for SARS-CoV-2 entry into the host cell is a cleavage site for the host cell protease furin at the S1/S2 boundary (32). Following production and maturation of the S protein in the host cell, furin cleavage at the S1/S2 site in the Golgi apparatus results in the two subunits being non-covalently associated to perform the different functions of attachment and virus-membrane fusion (**Figure 2**). Furin proteases are ubiquitously expressed in humans, resulting in enhanced tissue tropism and pathogenesis for SARS-CoV-2 (33). Another cleavage event occurs at the target host cell. Upon receptor binding through S1 and conformational changes, a S2' cleavage site within the S2 subunit becomes accessible for cleavage at the membrane of an infected cell (**Figure 2**). Cleavage of the S2' site by cellular proteases is a pre-requisite for entering host cells as it exposes the fusion peptide and initiates membrane fusion. The type II transmembrane serine protease (TMPRSS2) is the main host protease to prime the S protein for cell entry by cleaving the S2' site (34). Mediated by conformational changes of the S protein, the exposed fusion loop is inserted into the host cell membrane and the viral

and cellular plasma membrane fuse, through which the RNA-containing nucleocapsid complex enters the host cell (23, 35). Besides SARS-CoV-2, also MERS-CoV and HCoV-OC43 have a S1/S2 furin cleavage site, however, other coronaviruses, including SARS-CoV, lack such a site (36). These coronaviruses, however, harbor other sequences that can be cleaved by the proteases at the target host cell (37-39). Studies have shown that additionally to using TMPRSS2-mediated fusion at the cell membrane, some coronaviruses, including SARS-CoV as well as SARS-CoV-2, can also employ the endosomal route for entry, which is dependent on the host cell protease cathepsin L (CTSL), which performs cleavage at the S2' site in the endosome (40, 41). In that case, upon ACE2 receptor binding, the virus is first internalized through the endosome and fusion occurs between the virus membrane and the endosomal membrane (23). Omicron variants were even reported to have a higher dependence on the endosomal route for entry (42). Many other cofactors for viral entry were also identified, like for example neuropilin-1 or c-type lectins (43, 44). Likewise, studies have reported alternative receptors for SARS-CoV-2 attachment (45, 46).

Once coronaviruses have entered the host cell, they express the non-structural proteins (nsps) necessary to form the enzymatic complex for viral replication and transcription (**Figure 2**). From two large open reading frames (ORF1a and ORF1b) covering the 5' two-thirds of the coronavirus genome, first, two polyproteins are translated: pp1a (includes nsp 1-11) or pp1ab (includes nsp 1-16) (47). To generate the individual nsps, the polyproteins are proteolytically processed by the two internal viral proteases, located in nsp3 (48) and nsp5 (49). Nsp5, the main protease (Mpro) or chymotrypsin-like cysteine protease (3CLpro), releases all nsps downstream of nsp4. The replication-transcription complex that is formed, includes a helicase (nsp13), a RNA-dependent RNA polymerase (RdRp) (nsp12), processivity factors (nsp7 and 8), a single-strand binding protein (nsp9), a proofreading exonuclease and N7-methyltransferase (nsp14), and a 2'-O-methyltransferase (nsp16), and other cofactors (e.g. nsp10) (47). Recently, the mechanism of viral RNA capping, which is important for efficient translation and hiding the viral RNA from host detection, was elucidated in more detail (50). Mediated by the nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain of nsp12, nsp9 forms a covalent RNA-protein complex with the nascent RNA, a mechanism termed RNAylation, which enables further capping of the RNA.

From the virus genome, full-length negative-sense RNA is produced as a template for new positive-sense RNA, which is then used for translation, packaging into newly formed virus particles or further genomic RNA replication. Replication of new viral RNA is thought to happen inside double-membrane vesicles (DMVs), which arise by the transformation of endoplasmic reticulum (ER) membranes, and were shown to be induced by expression of nsp3, nsp4 and nsp6 (51-54). Although many details of coronaviral RNA synthesis inside DMVs remain to be elucidated, recently, molecular pores in the DMV membranes were

found, which could enable the trafficking of newly synthesized RNA to the cytosol for translation and incorporation into viral particles (55). Structural and accessory proteins are translated manifold from a nested set of subgenomic mRNAs. These subgenomic mRNAs derive from the 3'-proximal third of the positive-sense genome through a mechanism where minus-strand synthesis is discontinuous (47, 56, 57). Following the translation of structural proteins in the cytosol, the envelope proteins S, M and E are processed into their mature form in the ER and trafficked to the ER-Golgi intermediate compartment (ERGIC). The S protein is for example heavily glycosylated, which has implications for antibody recognition and infectivity, as the glycans on the S surface were shown to be necessary for efficient binding of S to the host receptor ACE2 (58-60). The envelope proteins, together with N-encapsidated genomic RNA, assemble into new virions at the ERGIC. The assembled virions then traffic to the Golgi apparatus, where furin-mediated S protein cleavage occurs (as described above for some coronaviruses like SARS-CoV-2). Finally, coronaviruses then exit the cell through exocytosis through the secretory pathway or lysosomal egress (61).

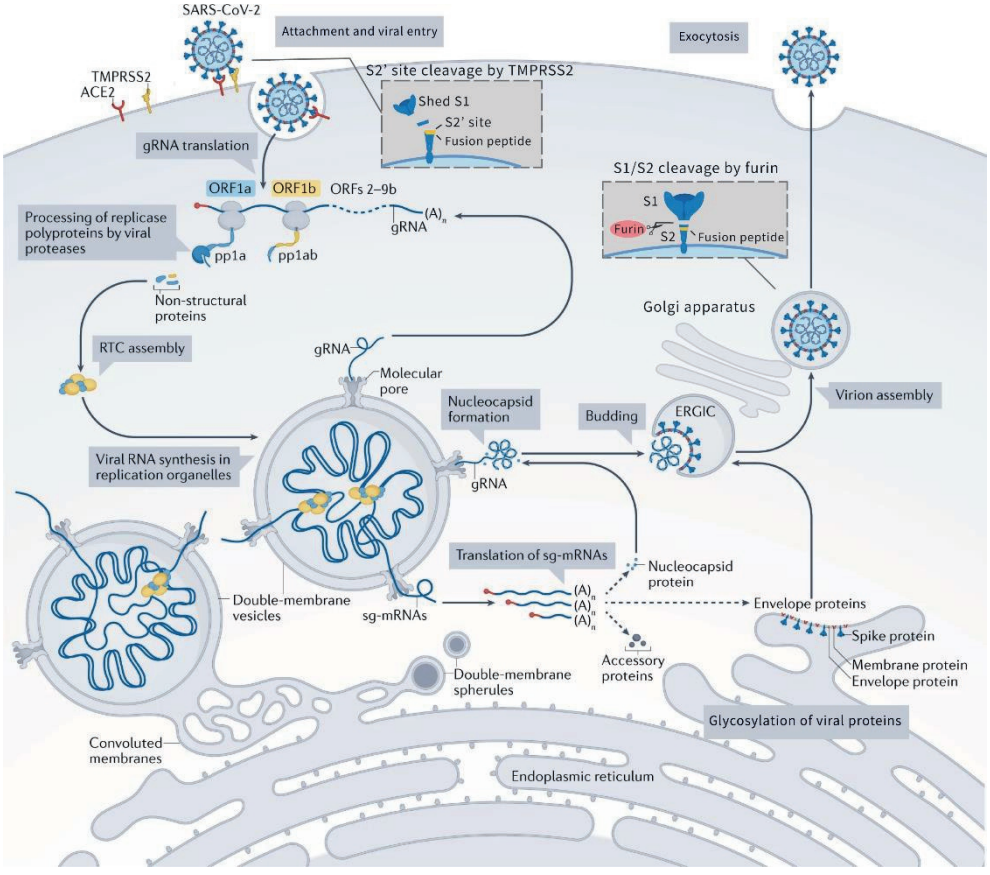

Figure 2: Schematic representation of the coronavirus replication cycle. Following receptor binding and virus entry into the host cell, the positive-sense genomic RNA is translated and processed to produce the non-structural proteins necessary for the replication-transcription complex (RTC). The figure shows the proposed model of RNA replication inside DMVs and the export of newly-made viral RNA into the cytosol through DMV membrane pores. Following the translation of subgenomic mRNAs in the cytosol, virus particles are assembled at the ERGIC. In the case of SARS-CoV-2, the S protein is pre-cleaved by the host protease furin in the Golgi apparatus. Adapted from (47) and (23) and reproduced with permission from Springer Nature. TMPRSS2: Transmembrane protease serine subtype 2, ACE2: Angiotensin-converting enzyme 2, pp1: polypeptide 1, gRNA: genomic RNA, sgRNA: subgenomic RNA.

Lung epithelium and coronavirus infection

The human respiratory tract is the point of entry for respiratory viruses like coronaviruses and spans from the nasal cavity to the lung alveoli. It is lined by the airway epithelium, which is composed of varying cell types depending on the anatomical location (62, 63). The airway epithelium is not only a physical barrier against invading pathogens, but the cell types also differ in their functions. In the tracheae and bronchi of the upper airway, the epithelium mainly includes ciliated cells, secretory goblet cells, club cells, and basal cells. Also other cell types, like ionocytes, tuft cells, and neuroendocrine cells, were described, which are less abundant (64). In the most distal area of the lung are the alveoli, where the exchange of oxygen and carbon dioxide happens. The alveolar epithelium is composed of alveolar epithelial type I cells (AEC1), which perform gas exchange, and their progenitors, the type II cells (AEC2) (65). AEC2 cells are important for generating pulmonary surfactant, a crucial mixture of lipids and proteins that is responsible for reducing surface tension in the alveoli and therefore preventing collapse at exhalation. Mucociliary clearance, which is the combined action of mucus secretion by goblet cells and the beating cilia of ciliated cells, actively transports particles, including pathogens/viruses, out of the upper airway (63). As a first line of defense, epithelial cells also initiate the innate immune response by production of cytokines and chemokines and subsequent recruitment of immune cells to the site of infection (64, 66).

In healthy individuals, the cellular composition of the epithelium differs throughout the airway, which causes a gradient of susceptibility to different respiratory viruses depending on their cell tropism (62). ACE2, the receptor that is utilized by SARS-CoV and SARS-CoV-2, as well as the protease TMPRSS2 are mostly expressed on ciliated cells and AEC2 (67-69), rendering them the primary target of initial infection. Also, MERS-CoV was found to infect AECs (70). Besides the infection of AECs in the lower respiratory tract by the highly pathogenic coronaviruses, SARS-CoV-2 and MERS-CoV were also reported to target non-ciliated cells in the upper respiratory regions (69, 71, 72). Studies have shown that in the

nasal epithelium SARS-CoV-2 displays a tropism for ciliated cells, while MERS-CoV preferentially targets non-ciliated cells, like goblet cells (73). HCoV-OC43 likewise infects primarily ciliated cells, while HCoV-229E targets non-ciliated cells, and both these common cold viruses primarily infect the upper respiratory tract (74). SARS-CoV-2 variants were first observed to evolve continuously with respect to their capacity to infect the respiratory epithelium, mainly through mutations in the Spike protein that led to enhanced binding to the ACE2 receptor and entry into the host cell (75, 76). Later, although the Omicron strains also displayed higher efficacy in binding to the ACE2 receptor (77), they were reported to be more dependent on the host cell protease cathepsin L and were less efficient in using TMPRSS2 (42). Earlier strains were more efficient at replicating in lung epithelium and infecting the lower airway, while subsequent Omicron variants have adapted to replicating in the upper airway, consequently yielding less severe respiratory symptoms, at least in healthy individuals (78, 79). Their adaptation to the upper airway, especially the nasal epithelium, was reported to be a possible reason for the observed increased transmission, alongside stronger affinity for the ACE2 receptor and increased immune evasion through changes in the antigenic structure of the spike (80, 81). Accordingly, studies that investigated the basic reproduction numbers for SARS-CoV-2 variants, showed that the Omicron variant is 3.8 times more transmissible than the delta variant (82) and infection rates in the population increased faster during the Omicron wave than with any other variants (83).

In patients with chronic lung diseases like asthma or chronic obstructive pulmonary disease (COPD), the epithelium changes structurally and functionally. The composition of airway epithelium cells in such chronic diseases can differ, and epithelial barrier function and immune responses were shown to be impaired (63). In the lungs of asthma patients, for example, more mucus-producing cells were observed than in healthy airways (84), and in asthma and COPD patients, there is an increased susceptibility to respiratory infections, as shown for rhinovirus, SARS-CoV-2, or MERS-CoV (85-87). Therefore, the presence of infection-promoting host factors, like receptors or proteases, on specific cells, as well as the proportion of those cells in specific anatomical locations, can determine susceptibility to and spread of infection. Understanding, which cells are infected, which host responses are elicited, and which factors influence infection kinetics, are important to develop treatment for patients with respiratory infections like COVID-19.

SARS-CoV-2 pathogenesis and immune responses

While common cold coronaviruses like HCoV-229E only cause mild symptoms in the upper respiratory tract of healthy individuals, infection with the potentially highly pathogenic SARS-CoV, SARS-CoV-2 and MERS-CoV can lead to severe symptoms also in the lower respiratory tract (88). Coronavirus disease 2019 (COVID-19), the disease associated with SARS-CoV-2 infection, can present as a variety of clinical symptoms, from asymptomatic infection to very severe disease, and the exact determinants for these differences are not known. In healthy young individuals, especially children, SARS-CoV-2 infection often is asymptomatic or causes only mild common cold symptoms (89, 90). However, in older individuals, people with underlying medical conditions, but frequently also in adolescents or young adults, COVID-19 can be life-threatening (91, 92). A study that analyzed all confirmed cases from the start of the pandemic in February 2020 in China, reported an overall case fatality rate of 2.3% (93), others reported that 3-20% of infected patients required hospital care (88, 94, 95). Over the course of the pandemic, following the implementation of vaccination and antiviral strategies, and the advent and predominance of the Omicron variants, the case fatality rate has decreased drastically, to below 0.3% in August 2022 (96). Although SARS-CoV-2 initially infects the respiratory epithelium, infection can lead to multi-systemic disease. Often an infection is accompanied by fever, cough, muscle aches, fatigue, and/or shortness of breath. In severe cases patients can suffer from acute respiratory distress syndrome (ARDS), which can greatly impact lung function and lead to tissue fibrosis, multi-organ failure, and death (88). Previously, such severe disease outcomes were also seen with SARS-CoV or MERS-CoV infection (97, 98). Over time it became clear that SARS-CoV-2 infection can also have long-term consequences and can cause quite unique post-infection symptoms. The loss of smell or taste, caused by olfactory dysfunction during the acute stage of the infection, can affect patients for a long time, and more seriously, “Post-COVID-19 condition” or “Long COVID” has developed in a few percent of infected people (99, 100). Long COVID is described as symptoms affecting the lung, heart, gastrointestinal tract, blood vessels, and nervous system, among others, which are a consequence of the infection, but remain present long after the acute infection has resolved. Possible underlying mechanisms include immune dysregulation, microbiota disruption, autoimmunity, blood clotting dysfunctionalities, endothelial cell abnormalities, and dysfunctional neurological signalling (100). This illustrates the wide impact that SARS-CoV-2 infection can have on the entire human body, with in some cases severe impact on the daily function and quality of life of patients.

During initial infection, the immune system plays a crucial role in combating respiratory viruses. As mentioned earlier, the epithelial cells are the first to initiate an innate immune

response to an invading pathogen (63). Intracellular sensing of viral RNA (dsRNA or RNA with a 5'-triphosphate group) by retinoic acid-inducible gene I-like receptors (RLRs) like MDA5 or RIG-I (101, 102) leads to interaction with MAVS, which induces phosphorylation of IRF3, ultimately resulting in transcriptional upregulation of the expression of type I and type III interferons (103, 104). Toll-like receptors on the cell or in endosomes, detect pathogen-associated molecular patterns (PAMPs) and likewise activate signalling kinase cascades that activate transcription of proinflammatory cytokines (105), type I interferons, and IFN-stimulated genes (ISGs) (104). Inflammasomes, like NLRP3, and cytosolic sensors other than RLRs were also described to trigger type I IFN and proinflammatory cytokine production upon sensing of SARS-CoV-2 (104, 106, 107). The binding of secreted IFNs to IFN receptors further stimulates the expression of interferon-stimulated genes (ISGs) whose products exert a range of antiviral effects (108). SARS-CoV-2 and other coronaviruses counter the innate immune response of host cells by several viral immune evasion mechanisms. They do so by expressing a number of proteins that (also) have immune evasion activities (104, 109, 110), and presumably also by preventing detection by hiding viral RNAs that can trigger intracellular sensing inside DMVs (53), and by modifying their RNAs to mimic the 5' cap structure of host mRNAs (103, 111). The interferon response was reported to be delayed following infection with highly pathogenic coronaviruses, which evade the innate immune response (112). When IFN response is initially blocked and delayed, unhindered virus replication can later lead to a strong and persistent IFN and proinflammatory cytokine response, which can cause hyperinflammation and long-term (immune) pathologies, as shown previously for SARS-CoV and SARS-CoV-2 (113, 114). Early on, studies have shown an association between high levels of IL-6, IL-1, IFNγ, TNFα, NF-Kb, and other chemokines, and high morbidity in patients infected with SARS-CoV-2 (104, 115-117). Systemic damage to tissue and severe consequences of a dysregulated immune response and hyperinflammation are well documented for COVID-19 patients (118), albeit not fully understood, also due to genetic and immunological differences between infected individuals. Age, immune status, and underlying medical conditions of patients are risk factors and determinants for disease outcome.

Human primary airway epithelial cell culture to study coronaviruses

To study coronavirus replication, host responses and pathogenesis, and to develop antiviral therapies, *in vitro* cell culture models are required. Most commonly, conventional monolayer cell cultures of immortalized or tumor cell lines are used, like Vero E6 (African green monkey cells) or lung epithelial cell lines like Calu-3 or A549 (119). Their use has

advantages, like low costs and minimal effort, as most laboratories have these cultures already in use. They are sufficient for initial drug screening purposes (120), where high-throughput screening is essential to reduce time and costs during drug discovery. However, for further drug evaluation, these simplified monocellular systems are often not sufficiently representing the infected host organism, which can affect infection characteristics and drug identification (121). Propagation of SARS-CoV-2 in Vero E6 cells for example, results in rapid virus adaptation and loss of the S protein's furin cleavage site (122), which can be prevented by using a lung epithelial cell line (123). There has been progress in the development of advanced *in vitro* models like organoids, organ-on-chip models, and air-liquid interface (ALI) airway epithelial cell culture (124), that better represent the tissue in question as it exists *in vivo*. Human primary airway epithelial cells that are cultured at ALI (HAE-ALI) have become a well-characterized model in the research of respiratory infections, for example for disease modelling or studying virus receptors, cell tropism, or immune responses (125). To develop ALI cultures, primary cells are isolated from donor lung tissue and grown on a membrane, where they are exposed to air on their apical side and cell culture medium on their basal side (126) (**Figure 3**). *In vivo*, the cellular composition can differ depending on the anatomical location in the respiratory tract or the age or disease of a given patient. To recapitulate different anatomical locations in the respiratory tract, cells from different locations can be cultured, like the nasal cavity, trachea, bronchi, or alveoli. Isolation of nasal epithelial cells is the least invasive procedure, performed by nasal brushing. The advantage of using primary cells instead of conventional immortalized or tumor cell lines is that the former differentiate into the different cell types and organization as present in the pseudostratified lung epithelium *in vivo*. Thus, differentiated HAE-ALI cultures contain basal cells, secretory cells, club cells, and ciliated cells (**Figure 3**). The secretory goblet cells also produce mucus, which is moved through the action of beating cilia (127, 128). It was shown that HAE-ALI cultures represent the *in vivo* epithelium transcriptome (129). In HAE-ALI cultures, the cellular composition of the modelled epithelium can differ depending on the individual donor involved (130), differentiation time (131, 132), as well as culture conditions (133). Furthermore, inflammatory disease states in the lung, which also affect the epithelial cells (134, 135), can be modelled and immune cells can be co-cultured with these cells (136). The epithelial cells of HAE-ALI cultures elicit immune responses that recapitulate those observed *in vivo*, as was shown in cultures of nasal, bronchial, or alveolar cells (137-139). During the SARS-CoV-2 pandemic, HAE-ALI cultures were extensively used to study infection (31, 71, 132, 140), host responses (139, 141), or the impact of antiviral drug treatment (142, 143). All human coronaviruses are able to infect HAE-ALI cultures, which renders them a good model for comparative studies between highly or low pathogenic coronaviruses. In

the search for antiviral drugs, a positive result in HAE-ALI cultures increases the likelihood that the *in vitro* efficacy of a drug will translate into animal models and the clinic.

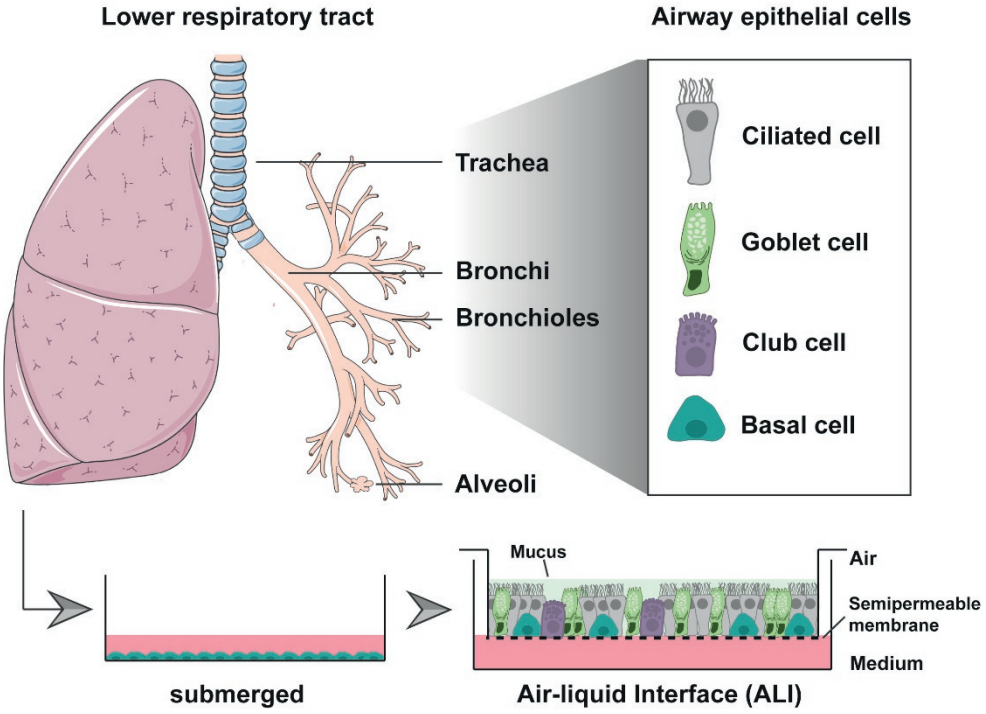

Figure 3: The lower respiratory tract contains trachea, bronchi, bronchioles, and alveoli, the latter being the region where gas exchange occurs. The epithelium of trachea and bronchi contains various cell types, primarily ciliated, goblet, club, and basal cells. Human lung tissue from these regions can be obtained to isolate primary epithelial cells, and culture them on transwell membranes at the air-liquid interface to obtain well-differentiated cultures. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

Antiviral drug discovery

Antiviral drugs and vaccines are the two key approaches to prevent or fight infectious diseases. During the SARS-CoV-2 pandemic, the development of vaccines succeeded at unprecedented speed, but still took over a year and demanded large budgets and effort. During this time, we would have benefited from having broad-spectrum antivirals at hand, to use in the clinic shortly post exposure or prophylactically in (local) outbreak settings.

However, although SARS-CoV and MERS-CoV emerged many years prior to the SARS-CoV-2 pandemic, no registered antivirals had been developed for clinical use against highly pathogenic coronaviruses.

There are two general approaches to antiviral drug development: one can either target the virus directly (direct-acting antivirals, DAA) or target host factors or pathways that the virus requires to replicate (host-directed antivirals, HDA) (144) (**Figure 4**). Direct-acting antivirals in principle are more specific, having activity against a more narrow range of viruses, but have a lower chance of toxicity. Due to virus evolution, there is more potential for the development of resistance against direct-acting antiviral compounds (145). Essentially, many steps of the viral replication cycle can be targeted, like virus entry, virus-cell fusion, RNA synthesis or other viral enzyme functions, or virus assembly (144). Host-directed antivirals, on the other hand, may have activity against a broad spectrum of viruses, while lowering the likelihood of resistance development, but present a higher risk of unwanted side effects (145). Therefore, a drug that has maximum selectivity towards the target, either a viral or host target, and minimal side effects on the host would be an optimal candidate.

The number of available antiviral drug treatments has slowly increased since the first antiviral drug was approved in 1963 (146). A review from 2002 listed more than 30 approved antiviral drugs (144), and a review from 2016 reported 90 approved drugs (145). However, these 90 drugs target a total of only nine human viruses, mainly the retrovirus human immunodeficiency virus (HIV), and DNA viruses like herpes simplex or human papillomavirus, and the RNA viruses influenza virus, respiratory syncytial virus and hepatitis C virus (HCV) (145). Since 2016, multiple other drugs that inhibit virus replication have been developed, targeting influenza virus (147), HIV (148), HCV (149), and most recently SARS-CoV-2 (150), all of them being DAAs. Successful drug categories, in general, are nucleoside analogues, protease inhibitors, entry inhibitors, fusion inhibitors, IMP dehydrogenase inhibitors, and neuraminidase inhibitors, to name a few (144, 145). Currently, the majority of approved antiviral drugs are DAAs, with the exception of entry inhibitors, like antagonists of the HIV receptor or co-receptor (151, 152), or the IMP dehydrogenase inhibitor ribavirin (153), although additional modes of action have been postulated for ribavirin (154). However, many HDAs that are targeting kinases or other signalling pathways are in preclinical development (155, 156). Furthermore, RNA-based or peptide-based therapeutics are new promising fields within the antiviral drug development landscape (157-159). Other treatment options do not inhibit virus replication, but modulate or stimulate the host's immune responses, such as pegylated interferon treatment, targeting inflammatory responses, and administering convalescent plasma or monoclonal antibodies (145, 160).

There are different approaches to the identification or development of new therapeutics. Antiviral drug discovery can be target-based, e.g. based on the known structure of a viral protein. Another way of finding candidate drugs is repurposing of already registered drugs (161, 162) (**Figure 4**). To identify hits from a number of newly developed compounds or a list of repurposing candidates, high-throughput screening is done. This can be done *in vitro* through phenotypic screening, where cell lines are used to evaluate drugs, for example, for their protective effect from cell death upon infection.

Recent advances in bioinformatics and artificial intelligence (AI) have broadened the options to screen for antivirals *in silico* (163), by either identifying new drugs or screening for potential repurposing candidates. AI can also assist in analyzing new drug targets, optimizing the drug development process, and predicting the properties of new candidate drugs, therefore de-risking drug development through identifying problems early and reducing the risk of failure. Repurposing of drugs offers the advantages of accelerating drug development, especially in an outbreak situation. Already existing knowledge of pharmacokinetics, pharmacodynamics, drug formulation, and safety profiles can decrease the time from preclinical assessment to clinical use. With regard to antiviral drugs, only ribavirin, which was reported to have different mechanisms of action (164), or the DNA polymerase inhibitor tenofovir have been previously approved by the FDA to treat more diseases than originally intended (145, 160). The RNA polymerase inhibitors remdesivir and the nucleoside analogue molnupiravir, which were already known as a broad-spectrum antivirals, were repurposed in the search for drugs to treat SARS-CoV-2 infection (143, 165). Other antiviral therapies that were successfully repurposed are immunomodulators, like corticosteroids, cytokine antagonists, or interferon therapies, which are not used to treat acute infections as they do not inhibit virus replication, but modulate the immune response.

Drug discovery can be divided into several stages: the phase of early drug discovery, which encompasses target identification and initial compound development; pre-clinical development; clinical development, which includes human trials; and regulatory approval and post-market evaluation (159) (**Figure 4**). Pre-clinical development encompasses several steps from initial target or drug candidate identification to clinical trials in human, and essentially entails investigation of pharmacodynamics, e.g. mode of action, efficacy and safety, and pharmacokinetics/ADME (adsorption, distribution, metabolism, excretion) and formulation of a drug (166). These properties can be evaluated partially *in vitro*, and *in vivo* in cell culture and animal infection models. During early drug discovery, high-throughput assays are performed in conventional monocellular cell lines, by treating infected cells with potential antiviral compounds, and with a simple readout that either shows protection from infection or inhibition of virus replication (167). Thus, a large number of chemical compounds can be screened, minimizing costs, manpower, and time. During pre-clinical

testing, the efficacy of a drug candidate can be confirmed in more advanced models, like ALI cultures, and moved into the next phases of drug development.

Drugs against SARS-CoV-2

Before the start of the SARS-CoV-2 pandemic, only two antiviral compounds against SARS-CoV or MERS-CoV had been tested in clinical trials or reported in case studies, which were the HIV protease inhibitors lopinavir and ritonavir (168) and the nucleoside analogue ribavirin (169-171). Studies reported reduced mortality for MERS-CoV-infected patients treated with lopinavir/ritonavir and interferon-β, and inconclusive results were reported for ribavirin. Furthermore, *in vitro* studies have identified multiple drugs possibly inhibiting these two zoonotic coronaviruses (172, 173). In the search for effective antivirals against SARS-CoV-2, researchers quickly screened drugs that were previously studied for their ability to block SARS-CoV and MERS-CoV, or other viruses (174). These drug repurposing efforts identified many potential inhibitors of SARS-CoV-2 replication. *In vitro*, those drugs might have yielded good test results, as reported for example for the RNA synthesis inhibitor favipiravir, the protease inhibitor lopinavir, or the anti-parasitic drug chloroquine (175-177). However, studies showing good efficacy *in vitro* did not translate into successful clinical trials (178, 179). This discrepancy of drug efficacy between *in vitro* and *in vivo* studies can result from the use of cell lines that lack relevance. Although chloroquine initially displayed promising results in Vero E6 cells (180), it was later shown that it does not protect human lung cells from SARS-CoV-2 infection (181). Remdesivir, the first drug that was approved for emergency use authorization (EUA), later received full authorization, albeit there is inconsistent evidence up until today about its efficacy and safety (182-184). However, studies did not always take into account the reduced efficacy of remdesivir when treatment is given to hospitalized patients with progressed disease, at a point where inhibition of virus replication does not lead to improvement (185). Remdesivir acts as a nucleoside analogue and inhibits the viral RNA-dependent RNA polymerase by terminating viral RNA synthesis (186). While remdesivir is administered intravenously, a derivative of remdesivir, VV116, is an oral drug that was recently approved for marketing in China (187). Other drugs were EUA approved but never fully approved, like molnupiravir, which, as a nucleoside analogue, inhibits viral replication by reducing the fidelity of viral RNA synthesis (188). Clinical studies evaluating molnupiravir as modestly efficacious led this drug to be approved for EUA by the FDA, however it was never approved further. While it is approved by the FDA, in Europe the EUA was later withdrawn (189, 190). To date only one orally given DAA has been approved as a treatment against SARS-CoV-2, with high quality of evidence

and strong recommendations from health agencies, which is ritonavir-boosted nirmatrelvir (marketed as Paxlovid by Pfizer), a compound that inhibits the main viral protease nsp5 (150, 184). Another promising inhibitor, which also targets the main protease and was approved for emergency use in Japan, is ensitrelvir (marketed as Xocova) (191). Both of these drugs were developed using target-based drug design. Notably, Pfizer scientists already had a compound in hand, that was developed against the main protease of SARS-CoV, thus substantially shortening the time of initial drug discovery (192). The fact that the main viral protease is indispensable for virus replication and is highly conserved across coronaviruses, makes it a good target for antiviral drug development and yielded one of the first successful SARS-CoV-2 antiviral treatment (49, 184). Potential inhibitors of the nsp3 papainlike protease have also been described (193, 194). Other studies have identified additional drug targets within the SARS-CoV-2 replication cycle, such as the nsp13 helicase (195), which is important for viral RNA synthesis, the nsp14 and nsp16 methyltransferases (196) or the nsp15 endonuclease (197), which is necessary for evasion of the host immune response (162). The nsp12 RdRp, like the main protease, also shows a high degree of conservation across coronaviruses (198, 199) and has potential as drug target for broad-spectrum active compounds, such as remdesivir. Furthermore, the nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain and nsp9, both essential for the capping of the viral RNA (200), are potential targets, and also the binding with associated nsp8 can be targeted (201). Further approaches are inhibition of entry, through blocking the spike protein (202) or the ACE2 receptor (203), or assembly, through targeting of the N proteins (160). Furthermore, interferon has been explored as a treatment option in multiple clinical trials, however with variable outcomes (204) and not resulting in approved treatment options. Many studies that employed the gene-editing tool CRISPR/Cas9 or small interfering RNA (siRNA) have identified host factors that are important for virus replication and therefore possible targets for drug development (205, 206). However, up until now, there are no successfully developed host-directed drugs that inhibit SARS-CoV-2 replication. There are approved HDAs that target other viruses, like hepatitis C virus (HCV) or human immunodeficiency virus (HIV), which are the infectious agents that most antiviral drugs were developed for prior to the SARS-CoV-2 pandemic (155). The efforts to develop HDAs against SARS-CoV-2 are ongoing with many small-molecule drugs in clinical trials and the therapeutic landscape evolving rapidly. This is not only important to treat SARS-CoV-2 infections but, considering the potential broad-spectrum activity of these compounds, also to prepare us for the next coronavirus outbreak. During the pandemic, it was quickly realized that COVID-19 can present itself with severe symptoms even after SARS-CoV-2 replication in patients has waned. Damage can be inflicted by a deregulated and exacerbated inflammatory response, triggered by SARS-CoV-2, leading to acute respiratory

distress syndrome (ARDS), pulmonary fibrosis, and multiple organ failure (207). Drug treatments reducing these disease symptoms, rather than inhibiting viral replication, have become important for therapeutic use. Host-directed drugs that are approved for the treatment of COVID-19 are immunomodulatory, like tocilizumab (IL-6 receptor antagonist) or baricitinib (JAK kinase inhibitor), which both suppress the inflammatory response (184). Also corticosteroids, like dexamethasone, were used early on for their anti-inflammatory effect (208) and are recommended for the treatment of systemic inflammation (209). Multiple trials have investigated the application of combination therapy, by simultaneously treating COVID-19 patients with an antiviral drug and immunomodulatory drugs (210). Optimal strategies for combating SARS-CoV-2 and its associated disease COVID-19 would involve drugs that inhibit virus replication and also exhibit a beneficial therapeutic effect, to prevent exacerbation of disease. Furthermore, for the treatment of COVID-19 patients, it is crucial to take into account the stage of disease (early in infection or advanced inflammatory disease) and the health status of the patient.

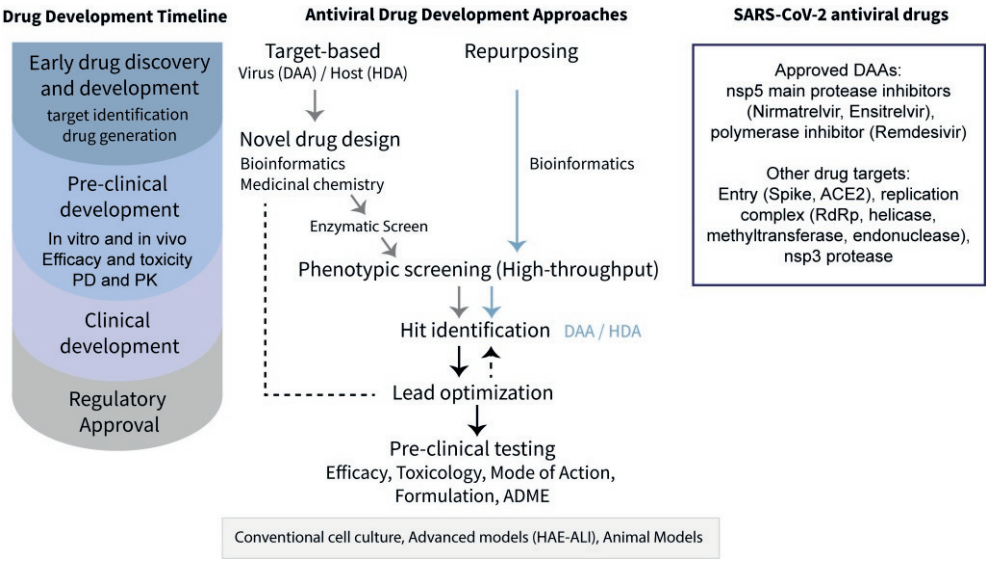

Figure 4: Antiviral drug development timeline and approaches. The currently approved antiviral therapies against SARS-CoV-2, and other main drug targets that are studied, are listed. Part of the figure is adapted from (160) and reproduced with permission from Springer Nature.

Outline of the thesis

The overall aims of this research project were to study coronavirus infection biology in lung epithelial cells and to contribute to the discovery of antiviral compounds. Therefore, primary human airway epithelial cells cultured at the air-liquid interface were used as an advanced model to recapitulate coronavirus infection of the human lung epithelium and host responses to SARS-CoV-2 and other coronaviruses. Host-directed antiviral approaches were evaluated by testing several groups of small-molecule compounds for their efficacy and broad-spectrum activity.

Understanding coronaviruses

In **Chapter 2**, HAE-ALI cultures were employed to set up and characterize an infection model with SARS-CoV-2. This cell culture model represents the respiratory epithelium very well and can reflect structural and functional changes as seen in individual donors or disease states. Therefore, it was used to study the impact of the presence of specific epithelial cells on SARS-CoV-2 infection. Differences in cellular composition based on anatomical origin, differentiation time or drug treatment and their effects on virus replication and viral cell-entry factors were investigated. In **Chapter 3**, the approach was broadened to compare infection with the potentially highly pathogenic SARS-CoV-2, SARS-CoV, and MERS-CoV and low pathogenic HCoV-229E and HCoV-OC43 viruses. RNA sequencing was used to identify differences in the host response to these viruses. The knowledge gained was used to identify possible antiviral compounds.

Targeting coronaviruses

Chapters 4 and 5 describe our efforts to repurpose and identify small-molecule drugs to combat SARS-CoV-2 and other coronaviruses. HAE-ALI cultures were utilized to detect antiviral activity in this advanced model. In **Chapter 4**, the compound R-Propranolol was studied for its anti-angiogenic and antiviral properties. By employing human lung endothelial cells, it was shown that propranolol blocks the upregulation of expression of angiogenic factors, which are induced by infection and potentially contribute to lung pathogenesis. R-propranolol also efficiently inhibited the replication of other highly pathogenic coronaviruses. In **Chapter 5**, several inhibitors of ER-resident α-glucosidase I and II were screened for antiviral activity against SARS-CoV-2. These comprised iminosugar compounds, which are studied as antivirals for decades, and cyclophellitols, a newer class of inhibitors. We identified 1,6-*epi*-cyclophellitol cyclosulphate as superior, as it most potently blocked ER α-glucosidase II activity and reduced virus infectivity. The broad-

spectrum activity of the antivirals identified in **Chapters 4** and **5** makes them interesting candidates to explore further for the treatment of coronavirus infections.

In **Chapter 6**, the research projects described in this thesis are discussed with regard to current literature. Additionally, new advances in SARS-CoV-2 drug development and the importance of the use of advanced cell culture models are discussed.

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