



Universiteit
Leiden
The Netherlands

The quest for broad-spectrum coronavirus inhibitors

Lima Leite Ogando, N.S.

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CHAPTER I

General introduction and Thesis outline

GENERAL INTRODUCTION

The word “quest” originates from the Latin *quaerere*. Since the 1300s, *quaerere* has evolved with the Medieval Latin, Middle English and Modern English languages and gave rise to the words “question” (from the imperative *quaere*) and “quest” (from the past participle *quaesitus*). In the 15th century, “quest” was applied as “a search”, and later was defined as “a number of events that occur to find” or “achieve something in the end”. Many are the examples of quests featured by writers such as John Ronald Reuel Tolkien in his master piece “The Lord of the Rings”. A quest has several components: a subject, a means to an end, challenges along the journey, at least one motif and an aim. Here, I will describe all the elements of my scientific pursuit, “The quest for broad-spectrum coronavirus inhibitors”, step by step, but not in a particular order.

The discovery of Coronaviruses - Walk the line (of history)

In 1996, the order *Nidovirales* was established by the International Committee on Taxonomy of viruses (ICTV) and united two families of positive-strand RNA viruses infecting vertebrates, *Coronaviridae* and *Arteriviridae* [1, 2]. Through the years, in particular following the advent of next-generation sequencing technologies and the conversion of virus taxonomy into a classification based on phylogeny, the nidovirus order expanded to (currently) 14 different families with many of them including only a single species [3]. The name ‘nidovirus’ was derived from a prominent common feature, the production of a nested set of subgenomic (sg) RNAs that share identical 5'- and/or 3'-terminal sequences with the genomic RNA (nest in Latin is *nidus*) [4, 5]. Additional common properties of nidoviruses are: a positive-sense, non-segmented linear RNA genome with a size ranging from 12-41 kb packaged into enveloped virions; a conserved genome organization including a large replicase in the 5'-proximal part of the genome; the presence of seven replicase domains that are critical to control genome replication and expression of viral proteins, 3C-like protease (3CLpro), RNA dependent RNA polymerase (RdRp), nidovirus RdRp-associated nucleotidyltransferase (NiRAN), Zinc-binding domain (ZBD), Helicase (HEL), transmembrane domain 2 (TM2) and transmembrane domain 3 (TM3); a conserved genome expression mechanism involving production of at least one subgenomic mRNA species; and the encoding of large polyproteins, with the one of the planarian secretory cell nidovirus (PSCNV) being exceptionally long, 13,556 amino acids (aa) [1, 5-10]. On the other hand, between the nidovirus families there are major differences in number, type and sizes of the viral proteins that make up the virus particle, resulting in variations in the morphology of the nucleocapsid and virion: spherical [11], rod-shaped [12], spherular with crown-like projections [13] or elongated rod-shaped [11, 14].

The best-studied family within the order *Nidovirales* is the family *Coronaviridae*, which currently incorporates the subfamilies *Letovirinae* and *Orthocoronavirinae*. The latter subfamily consists of four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* [15]. Some coronavirus (CoV) are important pathogens that challenge human public health or are of veterinary and economic interest. Whereas alphacoronaviruses and betacoronaviruses seem to exclusively target human and other mammalian host species, such as pigs, bats, cats and dogs, gammacoronaviruses and deltacoronaviruses infect a broader range of hosts including avian species (Table 1).

The first report on CoVs dates back to 1931, when a new acute and fatal respiratory disease in 2- and 3-year old chicken was described [16]. Only later, the microorganism responsible for this pathology, avian infectious bronchitis virus (IBV), was isolated using filtration techniques [17] and imaged by electron microscopy [13]. Subsequently, transmissible gastroenteritis virus (TGEV; [18]) in 1946 and murine hepatitis virus (MHV; [19]) in 1949 were isolated from swine and mice, respectively. The first full-length CoV genome sequence was reported for IBV in 1987 [20], followed by that of MHV a few years later [21, 22]. In 1965, the first human coronavirus, named B814, was isolated from a typical case of common cold and was cultured in human embryonic tracheal and nasal organ cultures [23]. A year later, a strain called 229E was isolated from students of Chicago University and cultured in human embryo kidney cells [24]. In order to study these viruses and fulfill Koch's postulates, healthy adult volunteers were inoculated with cultured virus. Curiously, in these studies the number of paper handkerchiefs used per day was taken as a marker for CoV infection [25]. In 1967, human CoV (HCoV) OC43 (OC stands for organ culture) was isolated from patient material and used for the first serological studies where serum of OC43-infected patients was tested for antibodies that would cross react with HCoV-229E [26]. Later, in 2003, HCoV-NL63 was isolated from a 7-months old child in the Netherlands (NL) [27, 28] and in 2004 HCoV-HKU1 was discovered in a 71-year-old Chinese man admitted to a Hong Kong (HK) hospital [29].

To date, only seven CoVs infecting humans have been characterized in cell culture, and all belong to the *Alphacoronavirus* and *Betacoronavirus* genera (Table 1). Four endemic human CoVs (HCoVs 229E, NL63, OC43 and HKU1) circulate annually and are associated with mild respiratory illness (common cold; [30]). The other three are zoonotic and (highly) pathogenic CoVs that emerged in the last two decades and were/are the cause of severe outbreaks in the human population: in 2002-2003, severe acute respiratory syndrome CoV (SARS-CoV; [31]) emerged in South East Asia; since 2012, and probably earlier too, Middle East respiratory syndrome CoV (MERS-CoV; [32, 33]) is causing small outbreaks in the Middle East; and severe

acute respiratory syndrome CoV 2 (SARS-CoV-2) is the cause of the on-going COVID-19 pandemic and was first discovered in December 2019 in Wuhan, China [34]).

Based on the abundant occurrence of related viruses in bat species, bats have been suspected to be a major CoV reservoir that has possibly given rise to several of the CoVs found in humans [35, 36]. In contrast, the detection of HCoV-HKU1 in rodents, the description of close relatives of HCoV-OC43 in rodents and phylogenetic analysis of their lineage (β CoV lineage A) suggest rodents as the possible original reservoir of these two viruses [37-40]. In general, peridomestic mammals may serve as intermediate hosts of CoVs. In the case of MERS-CoV and SARS-CoV, transmission to humans was attributed to an intermediate host species: dromedary camels [41] and civet cats [42], respectively. The origin of SARS-CoV-2 is still under investigation: it remains unclear if the epidemic was derived from a direct spillover of the virus from bats, or if a yet unknown intermediate host played a role [43]. Since 2003, the risk of another zoonotic CoV spillover into the human population has been predicted based (among others) on surveillance studies in which several SARS-like and MERS-like CoV sequences were extracted from bats [44, 45]. For example, the SARS-CoV-2 genome presents a high level of sequence identity (96.3%) with the bat CoV RaTG13 [46], while MERS-CoV is closely related to the bat CoVs HKU4 and HKU5 [33]. Various mutations and recombination events in different regions of the genomes of these zoonotic CoVs, mainly involving structural and accessory proteins, were identified and hypothesized to have contributed to cross-species transmission (reviewed in [47]). Furthermore, several human-related, pathogen-related and climate/environment-related factors (presumably) have promoted the dissemination of these viruses among humans worldwide. These factors include the easy access to international air travel and the exponential growth of the human population [48].

The recurrent spillovers of CoVs from animal reservoirs to humans, along with their ability to cross species barriers, indicate that future zoonotic transmission events are likely. This threat stresses the importance of studying zoonotic CoVs to understand their replication, control their spread and, ultimately, be prepared for the possible emergence of a new zoonotic virus. Preparedness for large outbreaks requires investments in prevention and surveillance programs, the development of vaccine platforms and the quest for broad-spectrum antiviral drugs. The main goal of this thesis project was to find inhibitors of CoV replication/infection. During the first 4 years of my work, the focus was on studying MERS-CoV, until February 2020, when SARS-CoV-2 took the world by surprise.

Table 1- Coronavirus and associated diseases

		Virus	Host	Respiratory inf.	Enteric inf.	Hepatitis	Neurologic inf.
Genus	α	canine coronavirus	CCoV	Dog		X	
		feline enteric coronavirus	FeCoV	Cat		X	
		feline infectious peritonitis virus	FIPV	Cat	X	X	X
		human coronavirus 229E	HCoV-229E	Human	X		
		human coronavirus NL63	HCoV-NL63	Human	X		
		porcine epidemic diarrhea virus	PEDV	Pig		X	X
		rabbit coronavirus	RbCoV	Rabbit		X	
		transmissible gastroenteritis virus	TGEV	Pig	X	X	
	β	bovine coronavirus	BCoV	Bovine	X	X	
		equine coronavirus	EqCoV	Horse		X	X
		human coronavirus HKU1	HCoV-HKU1	Human	X		
		human coronavirus OC43	HCoV-OC43	Human	X		
		Middle East respiratory syndrome coronavirus	MERS-CoV	Camels, Human	X	X	
		mouse hepatitis virus	MHV	Mouse, rat	X	X	X
		porcine hemagglutinating encephalomyelitis virus	PHEV	Pig	X	X	X
		rat coronavirus	RCoV	Rat	X		
		severe acute respiratory syndrome coronavirus	SARS-CoV	Civet cat, Human	X	X	
		severe acute respiratory syndrome coronavirus 2	SARS-CoV-2	Human, other?	X	X	
	γ	infectious bronchitis virus	IBV	Chicken	X		X
		Beluga whale coronavirus	BwCoV	Beluga whale	X		
		pheasant coronavirus	PhCoV	Pheasant		X	
		turkey coronavirus	TuCoV	Turkey	X	X	
	δ	porcine deltacoronavirus	PDCoV	Pig		X	

Inf., infection; Adapted from [49]. α , *Alphacoronavirus*; β , *Betacoronavirus*; γ , *Gammacoronavirus*; δ , *Deltacoronavirus*

Human diseases caused by Coronavirus infection – (Keep) Five feet apart

Transmission of HCoVs to susceptible hosts occurs mainly by the respiratory or fecal-oral routes of infection, with viral replication starting in epithelial cells. Each year, endemic HCoVs (229E, NL63, OC43 and HKU1) account for 15-30% of upper respiratory tract infections, mainly in the common high-risk groups: newborns, elderly people and individuals presenting comorbidities (reviewed in [50] and [49]). All of these HCoVs are distributed globally. Most of the infected people develop mild disease with common cold-like symptoms including fever, headache, malaise, sore throat and cough. The peak of disease symptoms is observed 3 to 4 days after infection and symptoms last for 7 days on average, up to a maximum of 18 days. Furthermore, HCoV-NL63 has been associated with acute laryngotracheitis (croup [51]). To date, only a few life-threatening infections with HCoV-229E have been reported in immunocompromised patients [52, 53]. Based on natural infections and studies involving healthy volunteers, reinfection with these HCoVs is relatively frequent, which suggests that infection does not provide long-lasting protective immunity [54].

In general, the pathogenic zoonotic CoVs, SARS-CoV, SARS-CoV-2 and MERS-CoV, can cause more severe disease that can progress into an atypical pneumonia after infecting the upper and lower respiratory tract. These CoVs have an incubation period from 1 up to 14 days with symptoms appearing typically between 3 and 7 days after infection. Patients present cold/flu-like symptoms, sometimes also with gastrointestinal manifestations such as watery diarrhea. The 2003 SARS-CoV outbreak was largely confined to Southern China and the Hong Kong region, affecting around eight thousand people (confirmed cases) with a fatality rate of about 10% [31, 55]. Early on, via international air travel, this pathogen was spread to 29 countries and regions. Human-to-human transmissions of SARS-CoV was mainly associated with hospital and household settings and human super-spreading events [56]. During this outbreak (in 2002/2003), the low number of asymptomatic infections facilitated the identification and isolation of infected people, followed by the implementation of strict quarantine protocols. Consequently, eradication of SARS-CoV was achieved in a relatively short period of time, around 6-8 months after the first cases [57]. SARS-CoV infection caused lower respiratory tract disease that could evolve into an atypical pneumonia characterized by acute respiratory distress syndrome (ARDS), which results in alveolar damage and oedema in the patients' lungs. Health follow-up of SARS-CoV survivors showed that after one year there was a fully recovery from the physical illness [58]. However, many patients reported concentration problems and psychological limitations.

During the on-going pandemic of SARS-CoV-2, more than 140 million cases (until April 2021) have been reported worldwide, with a case fatality rate of around 2%, corresponding to more

than 2.6 million deaths. The high number of asymptomatic SARS-CoV-2 infections (estimated to be one third of the cases) and easy human-to-human transmission through droplets and aerosols facilitated the spread of the virus (reviewed in [59]). Severity and prevalence of disease has been correlated with gender, age, obesity and the presence of other risk factors such as diabetes, immune- and hormonal-associated diseases [60-63]. The majority of people infected with SARS-CoV-2 develop mild or moderate coronavirus disease 2019 (COVID-19). Approximately, 5% of the COVID-19 patients experience severe symptoms, resulting in a need for intensive care including supplemental oxygen and mechanical ventilation. Strikingly, loss of smell (anosmia) is reported in many cases and it is due to viral replication in the sinonasal epithelium [64, 65]. For both SARS-CoV-2 and SARS-CoV infections, a triphasic pattern of disease is observed starting with mild respiratory and systemic symptoms (reviewed in [60]). The second phase is characterized by viral pneumonia derived from increased viral replication, and a third phase by the onset of an immunological and inflammatory response [66, 67]. Interestingly, children apparently are less susceptible to develop COVID-19, although in rare cases a multisystem inflammatory syndrome similar to Kawasaki disease was reported [68]. Still under evaluation, neurologic manifestations including impaired consciousness and acute cerebrovascular disease have been reported after patients recover from severe COVID-19, which raises some concerns about the future, taking into account the number of people affected by this disease. Furthermore, recent studies have described that, following asymptomatic or symptomatic SARS-CoV-2 infection, some individuals have prolonged/persistent symptoms for months, like fatigue, breathlessness, myalgia and insomnia, together referred to as Long COVID [69-71].

In 2012, MERS-CoV was first isolated from a 60-year-old patient from Saudi Arabia [32]. Since then, a series of small(er) outbreaks resulted in more than 2,500 confirmed cases with a 36% fatality rate. Concomitant with the seroprevalence of MERS-CoV antibodies in human populations across Saudi Arabian provinces, evidence of widespread infections of dromedary camels and asymptomatic human infections project a higher number of infections in humans than the actual known epidemiology [72-75]. Frequently, in 50-89% of MERS patients, mechanical ventilation is needed [76]. Inefficient human-to-human transmission and association of virus spread to close interactions between human and camels, crowded health care settings or closed family households contributed to a lower incidence of MERS-CoV cases (reviewed on [77]). Until now, 27 countries reported cases of MERS-CoV-infection, with 80% of infections occurring in Saudi Arabia. Although clinical features closely resemble those of SARS-CoV infections, many patients developed acute renal failure [32], ARDS, septic shock and multiorgan failure, resulting in higher fatality rate for MERS [78, 79].

In most cases, human CoV infections are not readily identified by clinical diagnosis because they usually cause mild disease and their symptoms cannot be easily distinguished from other respiratory tract infections (like those with rhinoviruses and influenza viruses). Early diagnosis has been critical to isolate infected people and avoid viral spread, during outbreaks and the on-going pandemic. Current diagnostics tools include detection of nucleic acid, antibodies or viral antigens and virus isolation/culture. Differential diagnosis through real time qPCR has been the standard method (with great sensitivity) to detect different CoVs in nasal swabs, saliva, gargling, sputum, deep tracheal aspirate, bronchoalveolar lavage or stool samples, and distinguish them from other respiratory pathogens (reviewed in [80-82]). Serological tests can determine the presence of IgM and IgG anti-CoV antibodies while antigen tests can detect the presence of CoV proteins like spike and nucleocapsid. These types of tests are predominantly used as retrospective diagnosis, or for the evaluation of immune responses to therapies and vaccination or for epidemiologic studies. Virus isolation is not routinely performed for diagnostic purposes, but it is essential to obtain isolates for characterization of specific pathogens and to support the development of its research, including therapeutic agents and vaccines. It can also be performed from nasal swabs, sputum and bronchial/alveolar lavage, although not always efficiently [83]. For surveillance purposes, NGS and subsequent quick data sharing are currently a good practice between clinicians, scientists and healthcare institutions to monitor virus evolution and attempt to control spread of new variants within and between countries/continents. Given the lack of effective anti-CoV drugs and yet unclear efficacy of vaccination campaigns against SARS-CoV-2, a lot of countries continue to impose or recommend the use of face masks and implement physical distancing guidelines similar to those used during the SARS-CoV outbreak in 2002/2003. In majority of cases, more restrictive measures were adopted like local or national lockdowns, implementation of a curfew to reduce public circulation and quarantine of symptomatic people with the intention to control the spread of this infectious agent.

The Coronavirus replication cycle - The perks of being a Coronavirus

Electron microscopy shows CoVs as roundish packed particles, with a diameter of approximately 100-125 nm, that have a surface layer of club-shaped projections [13]. These spikes (S) are homotrimers of a class I fusion glycoprotein, which are embedded in the viral envelope and provide a crown-like appearance, in Latin *corona* (Fig. 1A). As for all viruses, the CoV replication cycle can be divided in different steps: attachment and entry, uncoating, translation, genome replication, assembly and release. For attachment of virions to the cells, CoVs may use both proteinaceous and sialoglycan-based receptors (reviewed in [84]). The

binding to proteinaceous receptors is mediated through a specific receptor-binding domain (RBD) exposed at the surface of S1, one of the two subunits of S.

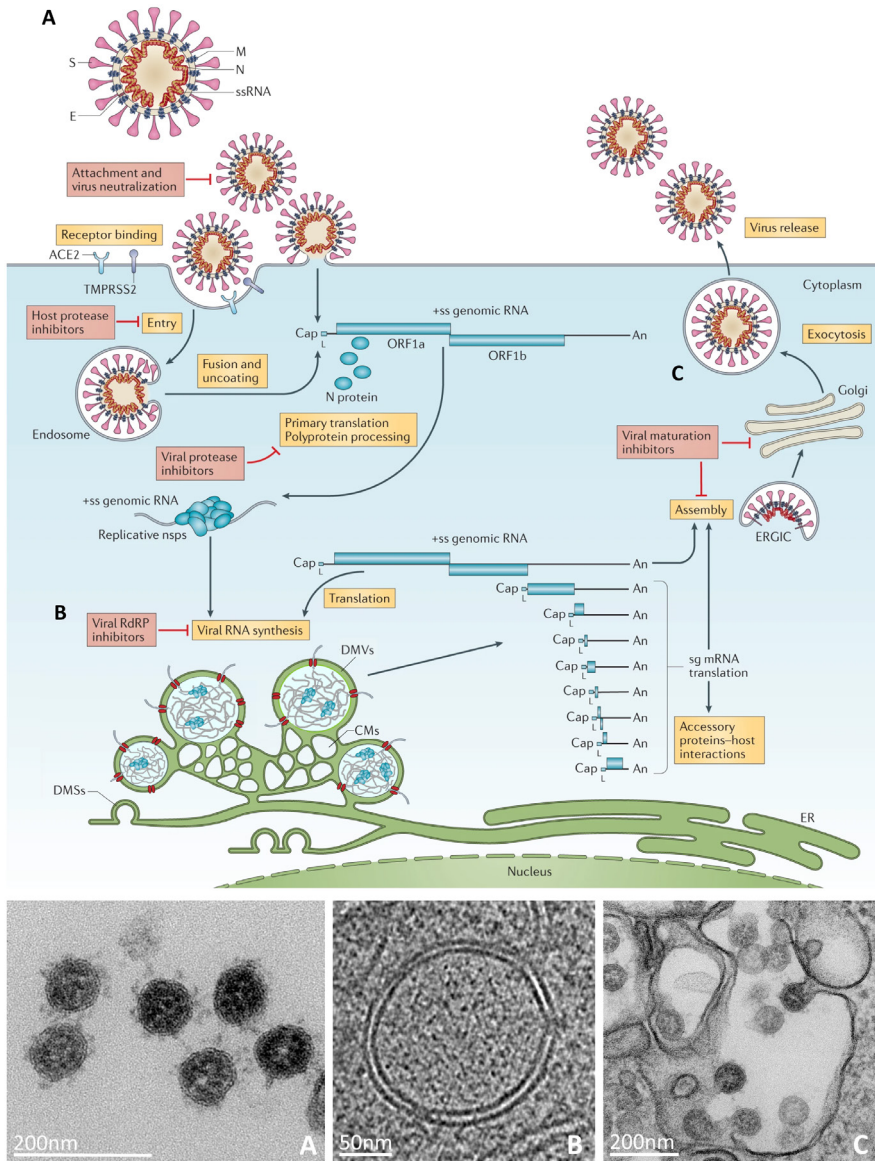


Fig. 1- Schematic representation of the coronavirus particle and replication cycle. At the bottom, electron micrographs of Vero E6 cells infected with SARS-CoV at 10 h p.i. and 8h p.i., respectively, showing extracellular viral particles (A) and virion assembly (C). Tomographic slice (7 nm thick) of a prefixed SARS-CoV-2-induced DMV with molecular pores embedded in its membrane (B). Images were used and adapted with permission from [85-87].

For several CoVs, the host cell receptor has been identified: aminopeptidase N (APN; for most of *Alphacoronavirus* such as HCoV-229E [88], TGEV [89] and porcine epidemic diarrhea virus, PEDV [90]), angiotensin-converting enzyme 2 (ACE2; for HCoV-NL63 [91], SARS-CoV [92] and SARS-CoV-2 [34, 93]), dipeptidyl peptidase 4 (DPP4; for MERS-CoV [94]) and murine carcinoembryonic antigen-related adhesion molecule (CEACAM; MHV [95]). Different CoVs from the four genera may use carbohydrates like sialic acids or sialosides as the main receptor (like BCoV and OC43), as attachment co-factors (TGEV and FeCoV) or as an alternative receptor to bind to the cells (presumably MERS-CoV) [96-99]. With the exception of MHV, the N-terminal domain of S1 mediates this interaction. The receptor specificity determines CoV tropism and, may contribute to pathogenicity taking into consideration the cellular receptor expression and tissue distribution in the host. In order to bind to the receptor, the S protein needs to be activated by proteolytic cleavage into two subunits, S1 and S2. A first cleavage occurs between S1 and S2 subunit resulting in a conformational change of the S protein, after which a second site is cleaved which is located within S2 subunit. This results in exposure of the so-called fusion peptide that mediates the fusion of the viral envelope with the host cell membrane. The timing of cleavage of the S glycoprotein is dependent on the virus species and is considered a barrier to zoonotic coronavirus transmission [100]. Both S cleavages can be accomplished by different host cellular proteases such as cathepsin L during endosomal entry, or by transmembrane protease serine (TMPRSS) 2, 6 and 11D at the plasma membrane [93, 101, 102]. For some CoVs, like MERS-CoV, S protein activation can be achieved by furin protease activity during virus egress, thus preparing the virus to infect the next cell [103, 104]. In contrast, the SARS-CoV S glycoprotein is likely cleaved only during virus entry while SARS-CoV-2 is thought to use different routes for S protein activation [105].

Following attachment, entry of viruses into the cells can occur via two pathways: release of viral genome into the cytosol after fusion of the viral envelope with the plasma membrane at the cell surface; or alternatively, virions can be taken up by endocytosis. In the latter case, acidification of the endosomal microenvironment results in fusion of viral and endosomal membrane, and subsequently, release of the viral genome into the cytoplasm (Fig. 1).

Remarkably, CoVs possess one of the largest known positive-sense RNA genomes (~30 kb) equipped with heavily structured untranslated regions at each end, a 5' cap structure and a 3' poly-A tail. Two thirds of the CoV genome are occupied by two large (briefly overlapping) open reading frames (ORF), ORF1a and ORF1b. The 3'-proximal third of the genome incorporates a series of ORFs that encode for the structural proteins [S protein, envelope protein (E), membrane protein (M), and nucleoprotein (N)], and the so-called accessory proteins. In the cytoplasm, ORF1a and ORF1b are translated by host ribosomes into the large replicase

polyprotein 1a (pp1a) and pp1ab. Proteins encoded by ORF1a are produced in larger quantities than those encoded by ORF1b due to a -1 programmed ribosomal frameshift that directs part of the ribosomes from ORF1a into ORF1b during genome translation [106, 107]. Subsequently, the replicase polyproteins are processed into 16, or in the case of *Gammacoronavirus* (like IBV) 15 individual non-structural proteins (nsps). This process is driven by 1 or 2 papain-like proteases (PL^{pro}) and a chymotrypsin-like or main protease (3CL^{pro} or M^{pro}) that reside in nsp3 and nsp5, respectively. Most of the polyprotein cleavages are performed by M^{pro} [108]. During the three decades that have passed since the first CoV genome sequences were obtained, enzymatic activities have been attributed to various nsps (reviewed in [85, 109, 110]) such as: RNA-dependent RNA polymerase (RdRp; nsp12), helicase (HEL; nsp13), exoribonuclease (ExoN; nsp14); methyltransferases (nsp14 and nsp16) and endoribonuclease (EndoU; nsp15). ORF1a mainly encodes proteins that perform proteolytic processing (PL^{pro} and M^{pro}), provide the necessary supporting functions for formation of replication organelles (RO), and supply co-factors for RNA-synthesizing enzymes. On the other hand, ORF1b encodes nsps that perform core enzymatic functions involved in RNA synthesis, fidelity control and post-transcriptional RNA modifications. Both ORFs encode nsps with accessory functions presumably involved in interactions with the host (nsp1) and host immune evasion (PL^{pro}, ADRP, EndoU and others). The 3'ORF proteins include the structural proteins and proteins that (presumably) modulate virus-host interactions or are associated with virulence (reviewed in [111, 112]).

Most of the nsps assemble into the viral replication and transcription complex (RTC) that uses positive strand genomic RNA (gRNA) as template for the replication of new gRNA and transcription of sub-genomic (sg) mRNAs through subgenome-length negative-strand intermediates. For certain CoVs, nsp1 is involved in modulation of the host cell translation machinery by interacting with the 40S ribosomal subunit [113, 114]. Following the expression of nsps, viral ROs are formed, probably triggered by the membrane association of specific nsps that have hydrophobic domains (nsp3, nsp4, nsp6). This leads to the abundant formation of paired membranes and double-membrane vesicles (DMVs) for which the endoplasmic reticulum (ER) is the likely membrane donor [115, 116]. The double-membrane vesicles (DMVs) have been identified as the site of viral RNA synthesis [116, 117] and they may provide a suitable micro-environment for viral RNA synthesis. Recently, it was discovered that, these DMV membranes contain hexameric molecular pores, including the large nsp3 subunit, that connect the DMV interior with the cytosol, where viral mRNA translation and packaging need to occur ([87]; Fig. 1B). Although further research is needed, this suggests that the pore may serve to export viral RNA from the DMVs, and perhaps also for the import of metabolites and

protein factors needed for RNA synthesis. Hypothetically, on the one hand, these double-membrane structures may concentrate the NTPs, RNAs and proteins necessary for RNA synthesis and, on the other, hide viral replication products, such as double-stranded RNA intermediates, from detection by the host's innate immune system [116].

Similar to all + RNA viruses, CoV RNA synthesis alternates between negative and positive strand RNA, with the nsp12-RdRp being the centerpiece of the RTC, together with its co-factors nsp7 and nsp8 [118, 119]. Associated with this complex is the nsp13-helicase to unwind dsRNA in a 5' to 3' direction [120-122] and nsp14-ExoN which can excise mismatching nucleotides incorporated in the RNA chain, thus serving as a proof-reading enzyme that improves the fidelity of RNA synthesis ([123-127] and reviewed in **chapter III** of this thesis). Besides the synthesis of copies of the full-length gRNA, the transcription process involves discontinuous minus strand RNA synthesis to produce subgenome-length minus strand RNAs that serve as the template for mRNA production [128, 129]. Along the CoVs genome, there are short conserved sequences known as transcription-regulating sequences (TRSs) located upstream of most of the 3' ORFs (body TRS) and near the genome's 5'-end (leader TRS). In a unique process, during negative-strand synthesis, the viral RdRp may stop at a body TRS and reinitiate synthesis at the TRS leader of the positive sense template [129, 130]. Base-pairing between leader and body TRS determines the stability of TRS duplexes. In the end, a set of negative-strand templates that possess a poly-uridylate tail and an anti-leader sequence are produced. Most of the sg mRNAs are polycistronic and their abundance controls the amount of the corresponding structural and accessory proteins relative to each other and to the nsps [131]. Generally, only the 5'-located ORF, which is absent in the following shorter sgRNA, is translated into protein [132].

Ultimately, two essential modifications are introduced on both sg- and genomic mRNA to mimic cellular mRNAs: a cap-1 structure at the 5' end and a polyadenylate tail at the 3' end. The capping mechanism involves four sequential reactions performed by the nsp13 RNA triphosphatase (TPase; [133]); a RNA guanylyltransferase (GTase), still to be defined/characterized; and two methyltransferases, the nsp14 (guanine-N7)-methyltransferase (N7-MTase; [134]) and the nsp16 2'-O-methyltransferase (2'O-MTase; [135]). The 5' cap-structure is important for translation as it is recognized by eIF4E, which is part of the pre-initiation complex that mediates the binding of ribosomes to mRNAs [136]. After sg mRNA synthesis, structural and accessory proteins are translated in the cytoplasm. The S, E and M proteins, the viral envelop components, are inserted into the ER and transit to the site of virion assembly, the ER-to-Golgi intermediate compartment (ERGIC). Moreover, newly made full-length gRNA is packaged by the cytosolic N protein (forming RNPs) and buds into the ERGIC. This results in

viral particles with a host cell-derived envelope containing the viral M, E and S transmembrane structural proteins ([137, 138]; Fig. 1C). In the end, virions are secreted from the infected cell by exocytosis. Recently, it has been suggested that egress of MHV and SARS-CoV-2 viral particles from intracellular environment may (also) occur via the lysosomal trafficking pathway [139]. In this way, virions may induce deacidification of lysosomes, and therefore avoid degradation of viral particles.

Coronavirus inhibitors - The good, the bad and the ugly

Despite all research efforts to create either prophylactic (prevent infection) or therapeutic (treat infection) options, there is no registered antiviral drug against any HCoV. One exception is remdesivir, which was authorized by health authorities for emergency use in United States, Japan and Europe for the treatment of hospitalized adult and pediatric patients with suspected or laboratory confirmed COVID-19. However, only modest clinical efficacy and no impact on the survival of COVID-19 patients treated with remdesivir was observed in a recent WHO trial [140] as also reported by others [141-144], probably due to the fact that the drug is usually administered to patients in a late(r) stage of serious COVID-19 disease. Based on the promising results of some small clinical trials performed in 2020, favipiravir has been also licensed for emergency use in certain countries including Japan, Russia, Malaysia, India and Thailand for the treatment of COVID-19 patients [145]. However, in some of these countries, it still remains under regulatory review.

Thus far, driven forward by the COVID-19 pandemic, vaccine development has by far outpaced antiviral drug discovery. The majority of vaccine development strategies is based on using the S protein as an antigen to elicit a potent neutralizing antibody response. Antibodies generated against S protein in recovered SARS patients are immunodominant and long-lasting in humans and animals [146-148]. Approaches previously used to develop CoV vaccines included DNA plasmids, nanoparticles, virus-like particles, viral vector preparations using adenovirus or vaccinia virus as platforms encoding viral antigens, chemically inactivated virus and live attenuated virus (reviewed in [149, 150]). In 2020, innovative solutions using mRNA technology resulted in the fast-track development and production of FDA-approved vaccines (reviewed in [151]). Although, no vaccines against SARS and MERS have been approved and most of them did not progress beyond Phase I clinical trials, four vaccines have now (March 2021) been approved for use against COVID-19 by the European Medicines Agency (EMA). These include mRNA vaccines encoding the S protein of SARS-CoV-2 from Pfizer/BioNTech and Moderna, and adenovirus-vectored vaccines encoding the SARS-CoV-2 spike glycoprotein from AstraZeneca and Janssen/Johnson & Johnson. Based on results in countries with

advanced vaccination programs, like Israel and the United Kingdom, vaccination clearly reduces severe disease and the number of hospitalizations. However, the impact on preventing transmission between humans and re-infection of vaccinated people is still under evaluation. It is also too early to know how long the protection derived from vaccination would last. Moreover, the circulation of new SARS-CoV-2 variants worldwide, carrying multiple mutations in the S protein, may affect the vaccine efficacy. Therefore, for a number of reasons, it remains critical to also pursue antiviral drug development for CoVs. Social and economic factors, including compliance by the public and cost-effective production/distribution, play an important role in successful vaccination campaigns, especially to achieve the threshold necessary to obtain herd-immunity. Moreover, not all individuals can receive vaccination such as young children, immunocompromised patients and other risk groups or vaccination may be less efficient. In such cases, antiviral therapy can provide treatment of illness at the onset of symptoms, and/or when vaccination-induced protection is incomplete. Antiviral drugs can be designed to target different viral components with broad-spectrum activity against multiple or perhaps all CoVs (pan-coronaviral activity). If already developed and tested, antiviral could be administered at the onset of a future outbreak of a novel CoV, when specific vaccines will again not be available immediately.

Strategies for anti-CoV drug design and screening

To identify potential virus inhibitors different approaches can be used: structure-based drug design using crystal structures or structure models of a target, and enzyme-based or infected cell-based high-throughput screening (HTS) campaigns. The computer-aided approaches can contribute to the (*in silico*) design of inhibitors targeting a specific site/pocket of a viral protein, and to predict its potential inhibitory effect. Understanding structure-activity relationships can help to improve compounds by designing new analogues or derivatives. Subsequently, enzymatic assays and/or infected cell-based assays should follow to obtain proof of activity. The viral load reduction assays with different read-outs, such as quantification of viral genomes or progeny titers, or measuring a reduction of virus-induced cytopathology, provide information on the antiviral activity of compounds. In parallel, cytotoxicity of compounds should be evaluated in non-infected control cells, by directly or indirectly measuring concentrations of cellular metabolites. Selection of resistant mutants obtained by culturing virus in the presence of increasing concentrations of compound can give insights about the compound's target and potential mode of action. To evaluate the specificity of an inhibitor and explore target inhibition, biochemical assays can be developed. These approaches are used to identify candidate drug compounds that can be broadly classified as

direct-acting antivirals, targeting viral components (Fig. 1), or host-directed antivirals, targeting host factors important for viral replication.

Instead of designing new molecules, particularly when a new pathogen emerges, the testing of antiviral drugs registered for use to treat other viral infections or the screening of libraries with drugs approved for treatment of other diseases can be used to try to repurpose already marketed drugs. The advantage of the repurposing approach is the available knowledge of the drug's pharmacodynamic and pharmacokinetic properties, as well as any potential side effects of the compound. Moreover, repurposing can substantially reduce the time and investments commonly required for drug design/development. Thus far, most compounds developed against RNA viruses are direct-acting antivirals. Usually, therapeutics with different targets are combined in order to minimize the chances of the rapid development of drug resistance, which is commonly associated with the high mutation rate and potential for rapid adaptation of RNA viruses [78]. When developing therapies, one should take into consideration the potential cytotoxicity or other undesirable side effects of drugs potentially affecting cellular processes/pathways. Therefore, it is critical to balance specificity and efficiency during drug development. The ultimate goal is to identify broad-spectrum inhibitors that can target multiple current and future emerging CoVs. So far, treatment options for human CoV infections and derived diseases can be divided into the following categories: neutralizing antibodies, entry/fusion inhibitors, viral protease inhibitors, inhibitors of viral RNA synthesis (predominantly nucleoside analogues), and immunomodulators.

Entry inhibitors

One step of virus cycle replication that can be targeted with inhibitors is the entry of the virus into host cells. This can be achieved, for example, by inhibiting receptor binding, preventing conformational changes in the S protein needed for fusion activity, or modulating the catalytic activity of cellular proteases that are needed to cleave the S protein to achieve successful viral entry. For this purpose, antibody-containing convalescent plasma can be obtained from recovered patients and administered to newly infected patients, which resulted in a viral load reduction. Similar results were observed for treatment of SARS-CoV [152, 153], MERS-CoV [154, 155] and SARS-CoV-2 [156] infections when corresponding convalescent plasma was used. Most of the antibodies in convalescent plasma target epitopes on the RBD region of the S1 subunit, inhibiting S binding to the cellular receptor. As the amount of antibodies that can be obtained by extraction from blood of patients (convalescent plasma) is low, the production of recombinant human antibodies was developed as an alternative, which is based on immunization of transgenic mice and the cloning of variable regions or immortalization of

convalescent S protein-specific antibody-producing B cells [157]. Besides, monoclonal antibodies obtained by immunization of animals with viral antigens have shown high specificity and neutralization activity against different CoVs (reviewed in [158]) including SARS-CoV-2 [159].

Since the emergence of SARS-CoV in 2003, some laboratories have developed strategies to block the fusion step during CoV entry by targeting the heptad repeat 1 (HR1) and HR2 domains in the S2 subunit of the S protein. These regions play a key role in viral entry, by interacting with each other to form structures (six-helical bundle) that will bring viral and cellular membranes close together for fusion [160-162]. Although, this type of compounds is still under evaluation, a recently developed lipopeptide based on this concept was demonstrated to have pan-coronaviral inhibitory activity against the pseudotypes of 6 different CoVs [160, 163].

Another strategy to interfere with viral entry is to inhibit S protein cleavage by cellular proteases at the cell surface (TMPRSS2 inhibitors) or within endosomes (Cathepsin B and L inhibitors). Camostat mesylate is a synthetic serine protease inhibitor with broad-spectrum antiviral activity against SARS-CoV, SARS-CoV-2, MERS-CoV and HCoV-229E in cell-based assays. It also improves survival of SARS-CoV-infected mice [164-167]. Interestingly, only when used in combination with E-64d, an inhibitor of cathepsin B/L, complete inhibition of SARS-CoV-2 replication was observed [164]. This suggests that different entry routes can be exploited by SARS-CoV-2 [168]. As an alternative to camostat, nafamostat has been reported to efficiently inhibit SARS-CoV-2 entry at low nanomolar concentrations in lung-derived human Calu-3 cells and is currently under evaluation in clinical trials with COVID-19 patients. Compounds that prevent endosomal acidification, like ammonium chloride and the FDA-approved anti-malaria drug chloroquine/hydroxychloroquine, were also reported to have an inhibitory effect in cell culture and/or in animal models during MERS-CoV, SARS-CoV and SARS-CoV-2 infections [169-171]. However, other reports demonstrated that the inhibitory effect of this compound against SARS-CoV-2 was dependent on the cell line used [168]. Moreover, clinical trials revealed inefficiency of hydroxychloroquine to reduce the SARS-CoV-2 load in patients, which suggests that inhibition of the endosomal pathway is dispensable for efficient viral infection (revised on [172]).

In cell culture, suramin, another well-studied antiparasitic drug, has been shown to have antiviral activity against SARS-CoV-2 [173] and other RNA viruses such as Zika virus (ZIKV), chikungunya virus and herpes simplex virus type 1 [174-178]. Follow-up studies in animal models have been started to evaluate potential routes of administration. This is one of the drug repurposing efforts made in the course of the SARS-CoV-2 pandemic [179].

Viral protease inhibitors

Most proteins that are involved in viral replication are potential targets for drug design or development. Since the ORF1a-encoded viral proteases are indispensable for CoV replication, M^{pro} and PL^{pro} have both been targeted using synthetic small-molecule or peptide-like inhibitors and natural molecules, especially after SARS-CoV-2 emerged [180]. This includes known inhibitors for other proteases and newly designed inhibitors targeting the active-site of CoV proteases or other protein regions important for folding and stability. Based on the crystal structures available [180, 181], targeted screenings have been performed with part of the compounds being further characterized using enzymatic and cell-based approaches [182-185]. Structural differences between SARS- and MERS-CoV PL^{pro} account for the narrow-spectrum activity of inhibitors, whereas the high conservation of key M^{pro} residues involved in substrate recognition promotes the activity of inhibitors against multiple CoVs, at least *in vitro* [184, 186]. However, despite the amount of *in silico* and *in vitro* screenings, only a few compounds have been tested *in* animal models. Compound GC376, an M^{pro} inhibitor, demonstrated high potency against FIPV with capacity to reverse disease progression of severely ill cats [187]. Interestingly, this compound and some of its derivatives presented high affinity to proteases of other CoVs, including MERS-CoV, SARS-CoV and SARS-CoV-2. Two other compounds, designated 11a and 11b, exhibited efficient antiviral activity against SARS-CoV-2 in cell culture and good pharmacokinetics in animal models [188, 189]. The compound PF-00835231 displayed broad antiviral activity against different CoV subgenera at low nanomolar to picomolar concentrations and is now in Phase I clinical trials for COVID-19 treatment [190, 191]. Furthermore, combinations of protease and RdRp inhibitors have been tested against SARS-CoV-2 replication to prevent viral resistance [190].

Nucleoside analogues

In order to develop broad-spectrum antivirals, targeting the virus-encoded polymerase of different RNA viruses (such as hepatitis C virus (HCV), HIV, Ebola, Zika and influenza) has been a key strategy. The compounds used mainly are nucleoside analogues that can compete with endogenous cellular nucleoside pools for being incorporated into the RNA chain during its synthesis. Subsequently, elongation of the RNA chain can be abrogated or the introduction of mismatches can lead to an enhanced mutation rate, potentially resulting in ‘error catastrophe’ and reduced viral fitness [126, 192, 193]. As indicated before, lately, the most-studied nucleoside analogue with anti-CoV activity has been remdesivir, a prodrug of a monophosphoramidate adenosine analogue with efficient inhibitory activity against different HCoVs and other RNA viruses in cell culture and animal models [171, 194-197]. Other drugs

like favipiravir (T-705), N4-hydroxycytidine (NHC), ribavirin, sofosbuvir, AT-511, BCX4430, mycophenolic acid and penciclovir have been explored as alternatives *in vitro*, *in vivo* and in clinical trials. However, poor efficiency and severe side effects were associated with administration of ribavirin alone or in combination with interferon (IFN) α or IFN- β when tested in SARS and MERS patients (reviewed in [78]), presumably due to the high doses used [198-200]. Therefore, no clinical trials using solely ribavirin were pursued for COVID-19 patients. Nevertheless, this compound has been used to understand mechanistic properties of the CoV RTC *in vivo* [126] and *in vitro* [123, 201]. Efficient incorporation of favipiravir into RNA chains leads to an increased mutation frequency *in vitro* as observed with influenza, coxsackie and Ebola virus [202-204]. However, high concentrations of this compound are needed to have an inhibitory effect in CoV-infected cells. Sofosbuvir, a licensed therapy against HCV since 2015, displayed a high binding energy to the SARS-CoV-2 nsp12-nsp7-nsp8 RNA polymerase complex *in vitro* and abrogated RNA chain elongation [201, 205]. Despite the poor efficiency of sofosbuvir to protect cells against SARS-CoV-2-induced cytopathic effects [206], this compound currently is under investigation in animal models and clinical trials. The newest promising antiviral drug against CoVs is NHC, with proven inhibitory effect against different RNA viruses including HCV [207], Ebola [208] and VEEV [209], and betacoronaviruses in human airway cell cultures and mice [210, 211]. As it can be administered orally, NHC is a good candidate for HCoVs and it is currently awaiting clinical trials (mentioned in [212]). The pro-drug of this compound (EIDD-2801) is expected to act as a mutagen (more selective than 5-fluoro-uracil, 5-FU) that can be used prophylactically [213] and therapeutically [214].

Other viral protein targets

Several computational docking, *in vitro* and cell-based screening assays have been performed to find potential inhibitors of CoV enzymes encoded like the helicase, exoribonuclease and methyltransferases. Helicase inhibitors, that affect the unwinding and ATPase activities have been identified through compound screening [215]. Bananins and SSYA10-001 were demonstrated to be broad-spectrum CoV inhibitors at low micromolar concentrations [120, 216, 217]. A few small molecules were reported to inhibit the SARS-CoV nsp14 and nsp16 methyltransferases, such as S-adenosyl-L-homocysteine, sinefungin and aurintricarboxylic acid *in vitro* [218-222]. When testing these compounds in infected-cell assays, low efficiency and poor specificity was observed.

So far, of the CoV structural proteins, besides the S protein, only the E protein that is involved in viral assembly, morphogenesis and virulence in animal models can be efficiently blocked by small-molecule inhibitors. Hexamethylene amiloride is hypothesized to interfere with the ion

channel activity of the E viroporin of SARS-CoV, HCoV-229E and some animal CoVs [223, 224]. In order to target this and other non-structural, structural and accessory proteins, the use of RNA interference (RNAi) that complement mRNA strands for degradation in infected cells have been studied. Still, this mechanism reveals a narrow spectrum and needs investment on optimal delivery to be approved for use in humans [225, 226].

Host factor-targeting inhibitors

Targeting host factors that can modulate viral replication, which takes place in the cytoplasm, has also been explored as a strategy for drug design/development. CoVs employ different mechanisms to disguise their presence from pathogen recognition receptors by interacting with molecules involved in innate immune responses or by compartmentalizing their activities in host-derived platforms (DMVs, RO and ERGIC). During CoV replication, type I IFN production is limited or delayed in most infected cell types, like human airway cells, fibroblasts, and organoids [227-230]. Interestingly, CoVs are susceptible to different types of IFN treatment *in vitro*. Therefore, treatment involving the direct administration of IFN or molecules that can stimulate IFN production, such as corticosteroids, poly-I:C (synthetic analogue of dsRNA) and nitazoxanide, have been widely tested. Recombinant type I IFN inhibits SARS-CoV, MERS-CoV and SARS-CoV-2 replication in infected cells [231-233], with the latter being more sensitive to lower concentrations of IFN- α [86]. Surprisingly, no significant effect on the clinical outcome was observed with IFN- α 2a, IFN- α 2b or IFN- β -1a treatment or in combination therapies with ribavirin or with lopinavir-ritonavir when administered to SARS or MERS patients (reviewed in [78]). Reports on poly-I:C (commercially named Hitonol) demonstrated increased survival rates when administered 21 days up to 24 hours before infection of mice with a lethal dose of SARS-CoV [234, 235]. Besides being a potential prophylactic option, it could be used as therapeutic treatment if given early in infection. Nitazoxanide is a synthetic nitrothiazolyl-salicylamide that induces IFN- α and IFN- β production with broad-spectrum activity against different RNA viruses including human and animal CoVs [171, 236, 237]. However, its activity against human-pathogenic CoVs has yet to be fully determined in animal or clinical studies [238].

Corticosteroids such as dexamethasone were widely used during the SARS-CoV epidemics. Some studies reported a positive impact on the oxygenation index [239, 240], while others claimed that it resulted in prolonged viremia and had serious side effects [241, 242]. As a consequence, it was used as a last resource in the treatment of MERS patients [79]. In the case of SARS-CoV-2, it has been considered as a standard of care for COVID-19 patients with severe disease and who required mechanical ventilation [243, 244]. Overall, despite the

potency of IFN and its modulators as antiviral agents, it needs to be taken into consideration that it can potentially contribute to prolongation of viral clearance, boosting the inflammatory response and consequently aggravate the associated (serious) side effects when administered late in infection, i.e. phase 3 of SARS-CoV-2 infection, cytokine storm.

CoVs can modify intracellular membranes, and hide their RTCs within double membrane vesicles where dsRNA replication intermediates are produced [115, 116, 245]. Compounds like K22 can inhibit DMV formation of a broad-range of human and animal CoVs in cell culture [246, 247]. Although, resistance culturing of HCoV-229E in presence of K22 resulted in the appearance of nsp6 mutations and suggested a potential mode of action of this inhibitor, no further studies were performed to pursue its mechanism of action nor was it explored further as an antiviral strategy.

THESIS OUTLINE

The main focus of this thesis is the search for compounds with an inhibitory effect against coronaviruses, mainly MERS-CoV. In **chapter I**, a brief introduction to the general molecular and structural biology of CoVs is provided. The importance of the quest for inhibitors against these agents is emphasized by describing the pathogenesis and epidemiology of CoVs.

In the past decades, a lot of the studies in our laboratory have been dedicated to the fundamental biology of virus replication, using *enzymatic and cell-based assays*. Frequently, this was done in collaboration with other departments or institutes that shared their expertise in different fields, e.g. electron microscopy, proteomics or structural biology. The emergence of SARS-CoV-2, during the course of this project, obliged us to apply our skills to investigate this new agent. Part of this teamwork was the development of a toolbox to study the replication kinetics, rapid adaptation and cytopathology of SARS-CoV-2 in cell culture, which is described in **chapter II**.

Analysis of CoV genomes highlights the conservation of certain domains and proteins across the members of this family. In order to develop broad-spectrum therapies against these viruses, it is important to understand the role of essential viral enzymes. In **chapter III**, the knowledge regarding the RTC, in particular focusing on nsp14 and its 3'-to-5' exoribonuclease (ExoN) activity is reviewed. The importance of this enzyme in CoV replication has been mainly studied using ExoN knockout mutants of MHV and SARS-CoV. ExoN mutants of both these viruses are viable but attenuated, and display an increased accumulation of mutations in their genome. Surprisingly, corresponding MERS-CoV and SARS-CoV-2 ExoN knockout mutants were not viable, as described in **chapter IV**. This highlights an unknown but critical role of nsp14 in CoV replication in cell culture, besides its presumed function as a proofreading enzyme. The nsp14 is a bi-functional replicase subunit that contains an N-terminal ExoN domain and a C-terminal N7-MTase domain. Structural analysis of SARS-CoV nsp14 demonstrated that the N7-Mtase has a structure that distinguishes this methyltransferase from the common viral and cellular Rossman-fold enzymes. In **chapter V**, residues presumably involved in N7-MTase activity were identified using computational analysis of the nsp14 structure and sequence of different β -CoVs. Next, selected residues were mutated to alanine and we evaluated the impact of these substitutions on nsp14 enzymatic activities and on viral replication/viability. This functional analysis provides insights into the pocket regions of nsp14 that could be targeted for drug design and the development of N7-MTase inhibitors.

Over the past years, many compounds were tested in our laboratory to check their potential inhibitory effect against CoVs. Different classes of inhibitors, which presumably targeted viral components directly or indirectly, affected host-virus interactions, or modulated host

responses before or during infection, were analyzed in cell-based assays. **Chapter VI** shows that 6',6'-difluoro-aristeromycin (DFA) blocks MERS-CoV replication in different human and non-human cell lines at low-micromolar concentrations. Insights into the potential mode of action of this promising nucleoside analogue were sought using cell-based assays and resistance culturing.

In **Chapter VII**, voclosporin (VCS), a host factor-targeting compound, was evaluated as a SARS-CoV-2 inhibitor. This compound can be used as a therapeutic alternative for cyclosporin A (CsA), tacrolimus (TAC) and other immunomodulators used in transplant patients, to whom certain treatments used for control of viral infection cannot be administered. In cell-based assays, VCS could inhibit SARS-CoV-2 load in Calu-3 cells more efficiently than TAC and with similar potency as CsA. This suggests that this compound should be evaluated in clinical trials for patients undergoing this type of therapy as a substitute of CsA or TAC as it can further suppress SARS-CoV-2 replication.

Finally, in **chapter VIII**, the findings described in this thesis are discussed in the context of our existing knowledge about anti-CoV research, and the problems that remain to be solved. Additionally, new discoveries related to the CoV RTC are summarized and discussed.

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