The quest for broad-spectrum coronavirus inhibitors

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CHAPTER VIII

General Discussion

PREFACE

Before the ongoing COVID-19 pandemic, researchers studying CoVs encompassed a small community, of whom the majority had been working in this field for decades [1]. This reality has changed since early 2020, when SARS-CoV-2 emerged and COVID-19 was declared a public health emergency of international concern by WHO. Many research groups specialized in different fields, including structural biology, chemistry and immunology, shifted their focus to CoVs and have been contributing their expertise to the accelerated development of our knowledge about these viruses, and more specifically SARS-CoV-2. These efforts translated into a *boom* of publications, more than 1000 per week, accepted for publication by journals or pre-printed in scientific databases [2]. Therefore, it is difficult to keep up with and summarize all the new discoveries in the CoV field, and any effort made to do so today (in May 2021) may be outdated or incomplete in a few months from now.

Coronavirus infection in humans can be asymptomatic, result in common cold symptoms, or cause mild to severe pneumonia. The development of severe symptoms is mostly associated with other comorbidities and infection with zoonotic CoVs. Over the past two decades, three zoonotic and (highly) pathogenic CoVs emerged: SARS-CoV in 2002, MERS-CoV in 2012 (more likely earlier) and SARS-CoV-2 in 2019. The mortality, societal impact and economic costs of the latter zoonotic CoV exploded and these unprecedented consequences emphasize the immeasurable value of solutions to prevent transmission of these viruses and treat those infected.

Before 2020, there were no registered drugs that could inhibit pathogenic CoV replication or treat associated diseases. Over the past year, some therapeutics for emergency treatment of COVID-19 patients and at least four COVID-19 vaccines were approved. However, these resources are not (immediately) available or suitable to be administered to everyone. Together with the (potential) problems caused by continuous virus evolution, this means that it is essential to continue the identification and development of antiviral drugs and vaccine research. The main objective of the studies described in this thesis was to characterize CoV drug targets and search for broad-spectrum CoV inhibitors. For this purpose, two strategies were used: (i) studying CoV replication to characterize potential targets for new compounds and (ii) performing phenotypic screening of previously developed compounds. Cell-based screenings were performed (as described in **chapter II**) using different classes of compounds including immunosuppressive and non-immunosuppressive derivatives of cyclosporin A, hits from FDA-approved drug libraries and molecules synthesized by collaborators such as fleximers (nucleoside analogues [3]) and SAH hydrolase inhibitors [4]. Subsequently, some

compounds that reduced the virus-induced cytopathic effect without being cytotoxic were characterized further in mechanistic studies, genotyping of drug-resistant mutants, followed by confirmation using reverse genetics. **Chapters VI** characterizes a new small-molecule inhibitor, DFA, with antiviral activity against MERS-CoV, while **chapter VII** describes the potential beneficial use of Voclosporin, a recently marketed compound to treat active lupus nephritis, which may also reduce SARS-CoV-2 viral loads. The latter is currently being evaluated in a clinical trial for the treatment of COVID-19 in kidney transplant recipients (KTRs), who are already receiving immunosuppressive treatment. The development of antiviral therapies requires understanding of coronavirus replication and its interplay with host cells. In **chapters III to V**, the in-depth characterization of nsp14, one of the replication and transcription complex (RTC) components, provides evidence for its importance for virus viability and fitness, while establishing that nsp14 might be a good target for drug design. In this Discussion (**chapter VIII**), new findings important to understand CoV molecular biology are highlighted. In addition, developments in antiviral research are summarized, describing potential targets for drug design and exploring the road to the future development of effective inhibitors of CoV replication.

ANTIVIRAL RESEARCH

As long as humankind exists, infectious diseases have caused suffering and claimed millions of lives. Over the past 50 years, the number of newly emerging infectious diseases appears to have increased and they are also spreading more quickly. Based on our records of outbreaks and epidemics, a large number of these events have been caused by RNA viruses infecting the respiratory tract, like influenza virus, Nipah and Hendra virus, hantavirus and various CoVs [5]. Lower respiratory tract infections are among the ten leading causes of death globally, according to the last WHO report [6]. Despite the burden of these viral diseases, our armamentarium of antivirals remains limited to about 90 drugs approved for the treatment of human infections caused by only 10 viral pathogens including influenza virus, HCV, RSV and HIV [7]. None of these drugs was specifically designed/developed to target CoVs. However, upon the emergence of SARS-CoV, MERS-CoV and SARS-CoV-2, some of these existing antivirals were tested [8, 9], which led to the discovery of compounds with broad-spectrum activity potential, like protease inhibitors and nucleoside analogues.

At first, in an epidemic/pandemic emergency situation, when there is no approved treatment available, the search for drugs is centered on the re-purposing of compounds that were originally licensed for use against other (viral) infections or to treat other diseases. Drugs that were withdrawn or abandoned, due to sub-optimal efficacy against their primary indication,

can potentially be repurposed as well. The advantage of using this strategy is the availability of knowledge regarding the safety, pharmacokinetics, potential side-effects, optimal formulation and dosage of a drug. This information (supposedly) accelerates the process of drug approval that is time-consuming and costly. To date (May 2021), more than 2400 clinical trials have been initiated since the emergence of SARS-CoV-2, to test single compounds or drug combinations for the treatment of COVID-19 patients [10]. These trials include antibodies and several classes of small molecules, such as existing antivirals, natural molecules, antiparasitic and antibacterial drugs, and immunomodulators that inhibit CoV replication in infected cells and, for some, also in small-animal models.

Learning by trial and error: the importance of appropriate models

At the start of the pandemic (March 2020), one of the first compounds proposed for the treatment of COVID-19 was hydroxychloroquine, a less toxic derivative of chloroquine. This drug was first synthesized in 1946 and has been used as an antimalarial therapy. The broad-spectrum antiviral activity of these drugs against different CoVs (including MERS-CoV, SARS-CoV and HcoV-229E) and other RNA viruses *in vitro* [11-14] raised interest to immediately investigate its inhibitory effect in SARS-CoV-2 infected cells [15, 16], as also presented in **chapter II**. Based on the promising *in vitro* data, many clinical trials with hydroxychloroquine were started and the FDA even temporarily recommended its use in COVID-19 patients in March 2020. However, not much later both FDA and WHO advised against its use based on the risk of developing dangerous side-effects and the meta-analysis of randomized controlled trials that demonstrated a lack of efficacy to reduce progress of infection, morbidity and hospital admissions [17]. The presumed mode of action of these drugs relies on the increase of endosomal pH by the capturing of protons (reviewed in [18]). As one of the entry pathways used by CoVs is endocytosis, it was hypothesized that these drugs would prevent the fusion of viral and cellular membranes and thus the release of the viral genome into the cytoplasm. The inhibitory effect of chloroquine and hydroxychloroquine that was originally demonstrated in cell-based assays using Vero cells (monkey kidney cells) could however not be reproduced when human lung cells or more complex cell cultures like organoids were used [19, 20]. The main reason for this discrepancy is that the SARS-CoV-2 Spike (S) protein, which mediates viral entry, can be activated by different proteases that are differentially expressed in different cell lines. In some cell lines, like Vero cells, viral entry is activated by the endosomal-pH-dependent cysteine protease cathepsin L, while in lung cell lines, which present low expression of cathepsin, cell entry is dependent on other pH-independent proteases, like TMPRSS2 [21, 22]. Thus, in the latter cells, entry occurs by a different pathway, fusion at the cell surface, that is

not blocked by hydroxychloroquine. When tested in SARS-CoV-2 infection experiments using hamsters and non-human primate models, hydroxychloroquine provided no therapeutic benefit [20]. In general, no differences in clinical manifestations or viral load were observed between untreated and treated animals, even in a prophylactic set-up in which animals were treated at least 24 h before infection [23-25]. Randomized clinical trial results correlated with the pre-clinical studies, demonstrating that there was no difference between placebo- or hydroxychloroquine-treated COVID-19 patients [18].

The story of (hydroxy)chloroquine emphasizes that the use of appropriate cell-culture or animal models that properly mimic the *in vivo* conditions can be critical to investigate the inhibitory activity of compounds before following them up in clinical trials. Cell lines expressing the appropriate virus receptor (susceptible) and supporting complete viral replication (permissive) need to be used. When developing cell-based screening assays, the choice of cell line is primarily based on the degree of cytopathic effect caused by viral infection. Compared to MERS-CoV, unfortunately, not many cell lines support SARS-CoV and SARS-CoV-2 replication, unless facilitated by the artificial expression of co-factors such as TMPRSS2 [21, 26, 27]. Ideally, HAE cultures developed from nasal, tracheal or bronchial primary cells [28-30], lung organoids [31] or lung-on-a-chip [32] should be used to study CoVs, as these may more closely reflect the events occurring during infection in humans. However, the use of this type of tools comes with a high financial burden and technical challenges.

Timing and regime of antiviral treatment

The SARS-CoV-2 replication is often already declining by the time that symptoms develop and antiviral drug are then administered too late to have impact. Accordingly, coronavirus inhibitors targeting essential stages of viral replication would only be expected to decrease the severity of disease if they are administered early enough to reduce the viral load and spread within the body. Severe disease manifestations, such as acute respiratory distress syndrome, appear to be primarily driven by host-mediated inflammatory responses rather than ongoing viral replication [33].

One antiviral drug approved for emergency use against SARS-CoV-2 is remdesivir, an adenosine analogue with broad-spectrum activity against CoVs and some other groups of RNA viruses. Studies with MERS-CoV and SARS-CoV revealed the high efficacy of this compound in reducing viral loads in cell culture-based infection models and showed both its prophylactic and therapeutic efficacy in different animal models [34-36]. In some clinical trials for COVID-19, remdesivir treatment was reported to shorten the time to recovery in hospitalized adults [37, 38]. However, a large WHO clinical trial demonstrated that remdesivir treatment did not

reduce the duration of hospitalization or need for ventilation [17]. Moreover, remdesivir showed only modest clinical efficacy and no impact on the survival of COVID-19 patients, although this outcome may have been influenced by the timing of the start of treatment. So far, remdesivir has mostly been administered by intravenous infusion to hospitalized patients, (presumably) at a time point that the viral load has already decreased. If patients progress from the viral replication phase to the stage of elevated inflammatory response (cytokine storm), remdesivir cannot be beneficial as it is not an immunomodulatory agent. A combination therapy that tackles both viral replication and the inflammatory response could be ideal for the treatment of COVID-19 patients. Dual therapies using remdesivir and corticosteroids (like dexamethasome) and cytokine inhibitors (like tocilizumab EXO-CD24) are currently under evaluation in clinical trials.

To be administered as early as possible, remdesivir should become available in a different formulation than the one requiring intravenous injection, allowing its use during the asymptomatic phase immediately after diagnosis, or even prophylactically. According to remdesivir manufacturer Gilead, the development of an oral formulation is in progress. Two other nucleotide prodrugs, AT-527 and EIDD-2801 (Table 1), both available in pills, showed broad-spectrum activity against different CoVs in cell-based assays [39-41]. In addition, EIDD-2801 was demonstrated to have a potent antiviral effect against SARS-CoV-2 in mouse models when administered by oral gavage before intranasal infection [42]. This shows that the compound can be metabolized and is distributed to the lungs of the animals. This promising result suggests that an oral formulation at least in this case allows sufficient bioavailability. As an alternative, taking into consideration that SARS-CoV-2 replicates well in the nose, intranasal formulations could be developed.

Some new things in the loop

The lack of resources for anti-CoV drug discovery before 2019 was related with the low incidence of SARS and MERS in the human population. Treatment options that demonstrated potent *in vitro* and *in vivo* antiviral activity against SARS-CoV, MERS-CoV or other CoVs were not prioritized for clinical development [9]. Also the lack of reliable animal models to prove antiviral activity efficacy stalled further evaluation of prospective compounds. For example, rhesus macaques were validated as a valuable model for MERS-CoV infection only in 2016 [43], when autopsies of MERS patients confirmed the pathology described during experiments with this non-primate model performed 3 years earlier [44, 45]. Overall, drug development efforts needed to start almost from scratch when SARS-CoV-2 emerged.

One of the main limitations of studying zoonotic CoVs is the requirement for biosafety laboratories of containment level category 3, which restricts the access to trained people and increases the costs of research. In order to circumvent this problem, non-live virus solutions like pseudoviruses, chimeric particles expressing envelop proteins from CoVs on the surface of a benevolent carrier virus, can be used for screening of entry inhibitors and evaluation of neutralizing antibodies [46-49]. Moreover, this tool has been applied to understand the mechanisms of viral entry, for example the role of the furin cleavage site (**chapter II**), and monitor current vaccine efficacy [46, 50].

All along the path of drug development, two main requirements need to be met: drugs should demonstrate a high degree of safety and strong inhibition of viral replication. The identification and study of compounds targeting viral components (direct-acting antivirals) or compounds targeting host factors, which may indirectly inhibit viral replication, have been widely explored using different (new) strategies. Technological advances like genome- and proteome-wide approaches have created opportunities to map virus-host interactions and explore their relevance for virus replication and pathogenesis [51-53]. These interactomes have highlighted pro-viral factors that can potentially be explored as a target for broad-spectrum inhibitors across different CoVs. Examples are the interaction of the ORF9b protein of SARS-CoV and SARS-CoV-2 with Tom 70, a mitochondrial import receptor [51, 54], and the effect on CoV replication of the knockdown or knockout of Sigma1 receptor [51], which has also been implicated in HCV and Sendai virus replication [55, 56]. However, high cytotoxicity is often related with the use of host factor-targeting inhibitors as they are more likely to also have deleterious effects on the host cell. To mitigate this risk, more specific and potent inhibitors need to be used at lower concentrations and/or should be used for shorter periods of time.

The fact that protein structures can now be obtained much more easily by cryo-electron microscopy (Cryo-EM) and can be used in advanced computational approaches [57] enhances the design of drugs with higher potential specificity for their target and helps to understand the interactions between inhibitor and target. Interestingly, different databases have been created to promote sharing of scientific information on developed molecules and structures of potential targets, which reflect the adaptation of research to the new technology and big data sharing: the international crowdsourced initiative COVID moonshot and the platform covid19dataportal are two good examples. The small number of drugs that currently meet the criteria for approval for COVID-19 treatment by health authorities underlines the large need for robust preclinical drug discovery programs. This involves not only the design and

development of new compounds (instead of repurposing), but also dissecting the mode of action of available compounds in clinical and pre-clinical studies.

THE CORONAVIRUS REPLICATION AND TRANSCRIPTION COMPLEX

The success rate of drug design will be importantly improved by understanding the molecular biology of CoVs in more detail. Since the writing of **chapter III**, the knowledge on the CoV RTC has greatly expanded. In particular the acquisition of cryo-EM-derived protein structures and the expanded NGS possibilities, including long-read sequencing, contributed to a better understanding of how the subunits forming the CoV RTC orchestrate RNA synthesis. The following paragraphs describe the latest mechanistic insights, their implications for virus fitness and how some nucleoside analogues target the RTC.

The art of copying large RNA genomes

The replication and transcription of all positive-stranded RNA viruses occur in the cytoplasm of the infected cell. Coronaviruses encode two large replicase polyproteins, pp1a and pp1ab, that are processed into mature nsps by viral proteases that are embedded within the ORF1a-encoded part of the polyproteins [58, 59]. Most of these subunits assemble into a ribonucleoprotein complex (replication and transcription complex, RTC) that produces different types of viral transcripts. Viral trans-membrane proteins modify cellular membranes and create a micro-environment ('replication organelle') for viral RNA synthesis with which the RTC is associated (reviewed in [60]). The CoV RTC includes a range of nsps that play different roles during RNA synthesis, such as the RdRp, helicase, exoribonuclease, methyltransferases and corresponding auxiliary co-factors. The functions of most of the CoV replicase subunits have been discovered and characterized using a combination of bioinformatics, biochemistry, structural biology and (reverse) genetics (reviewed in [61-63]). For a long time, a notable knowledge gap was the lack of a structure of the nsp12-RdRp the central player of the RTC. Technical challenges in obtaining stable and active nsp12 proteins/complexes prevented the acquisition of crystal structures and complicated the biochemical characterization of the CoV RTC. In 2003, a prediction of the structure of nsp12-RdRp was published [64], but only in 2019, Kirchdoerfer and Ward solved the structure of the SARS-CoV nsp12, bound to its essential nsp7 and nsp8 co-factors, using cryo-EM [65]. Since then, several additional structures of nsp12-nsp7-nsp8_2, complexed with RNA and/or nucleoside analogues or with other RTC subunits such as nsp9 and nsp13, have been reported [66-73]. These structures will be important tools during the further elucidation of the

molecular mechanisms underlying specific steps of viral replication, the design of new antivirals targeting the RTC and the characterization of their (potential) MoA.

The CoV nsp12 possesses at least two functional domains: the Nidovirus RdRp associated nucleotidyl transferase domain (NiRAN) located in the N-terminal part of nsp12 and the RdRp domain. The latter is organized in motifs A to G distributed across three subdomains, the so-called finger, palm and thumb of the RdRp structure [64, 74, 75]. These seven conserved motifs are involved in: template binding (motif G), nucleotide selection and binding, and catalysis at the active site, which is composed of motif A and C and supported by motifs B and D. The initiation mechanism (priming) of CoV RNA synthesis remains to be elucidated and both primer-dependent and *de novo* (primer-independent) mechanisms have been proposed. If *de novo*-initiation is used, an RdRp commonly employs specific structural elements to guide and position the initiating NTPs for catalysis, as for example demonstrated for HCV [76]. Some biochemical support for such a mechanism was obtained using SARS-CoV proteins [77, 78]. In contrast, if a primer-dependent mechanism is used, another factor than the RdRp is needed to deliver a primer. So far, based on biochemical assays, nsp8, nsp12-NiRAN and nsp9 have been proposed to play a role in priming, which therefore is a matter of ongoing studies and debate. Initially, the viral nsp8 subunit was reported to be capable of synthesizing short oligonucleotide primers that could be used by the nsp12-RdRp [77, 79, 80]. In arteriviruses, the NiRAN domain was shown to perform NMP-ylation, covalently bind nucleoside monophosphates (NMP) to itself (self-NMP-ylation), or to other viral proteins while releasing pyrophosphate [75]. Additionally, a preference for UMP and GMP was observed [75, 81]. More recently, its counterpart in CoV nsp12 was shown to mediate the transfer of NMPs (nucleotidyltransferase) to nsp9 [75, 81]. One of the proposed possible roles of UMP-nsp9 is the priming of RNA synthesis starting at the polyadenylated 3' end of the viral RNA. This initiation mechanism would bear resemblance to that used by picornaviruses, in which the uridilylated viral protein VPg serves to prime viral RNA synthesis [82]. This model is also supported by bioinformatic analyses revealing structural similarities between CoV nsp12-RdRp and picornavirus polymerases, suggesting involvement of their common G motif in primer-dependent initiation of RNA synthesis [64, 83, 84]. Recently, nsp8 was identified as an alternative substrate for UMPylation by nsp12-NiRAN [85]. In the same study, both *de novo* and primer-dependent mechanisms for initiation of minus strand RNA synthesis were proposed to be used by the nsp12-nsp7-nsp8$_2$ complex, although the functional significance of having alternative initiation mechanisms remains to be elucidated [85].

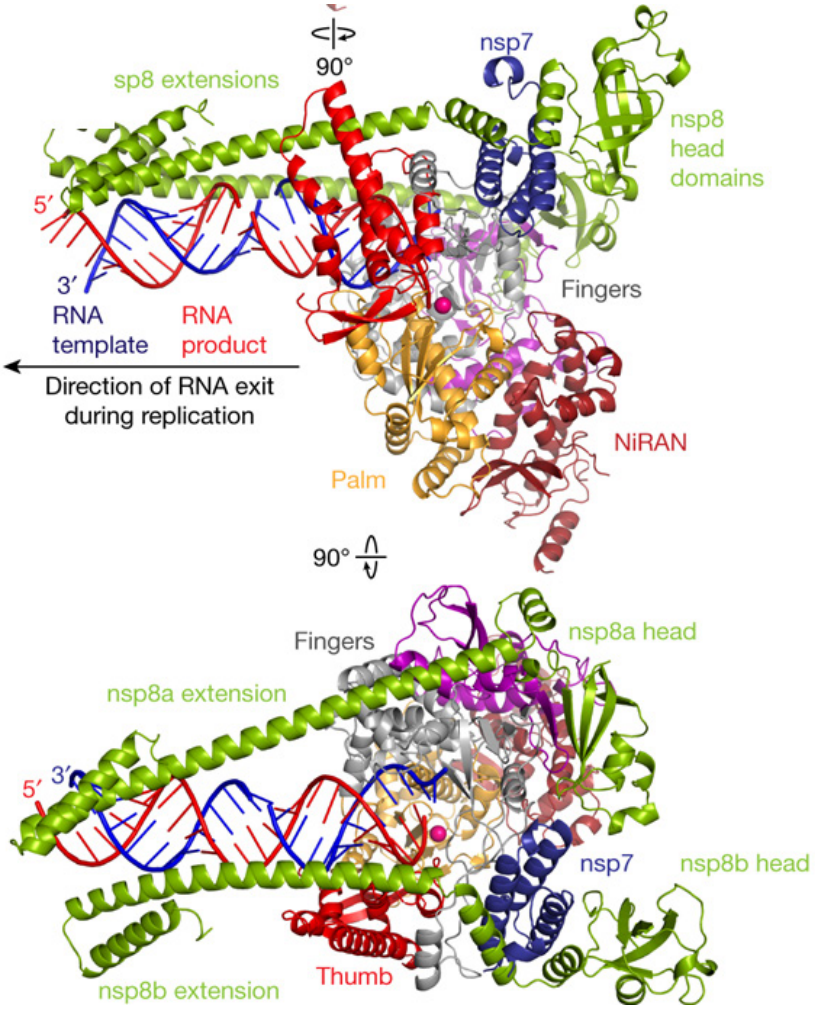

Fig. 1- Ribbon structure of nsp12-nsp8$_2$-nsp7 complex of SARS-CoV-2. RNA template in blue and RNA product in red. The nsp12 NiRAN and RdRp (fingers, palm and thumb) domains are depicted. Two positively charged 'sliding poles' extend from the RdRp and are formed by one nsp8 subunit in complex with nsp7 and one single nsp8 subunit. Interactions between positive-charged nsp8 residues and the emerging dsRNA during replication are thought to prevent premature dissociation of the replication machinery from its template. The sphere on top of the RdRp palm subdomain represents a metal ion in the active site of the RdRp. Re-used with permission from [69]

Minus strand RNA synthesis must start at the precise terminus of the RNA template or by priming at the genome-poly(A) tail junction, to ensure that the genetic information is copied completely [85]. Synthesis of new RNA proceeds by using +RNA strand as a template to produce a complementary full-length genome or sub-genome -RNA strand (as described in **chapter I**). In turn, these -RNAs strand then serve as a template for the synthesis of +RNA

strand molecules of both types (genome or sub-genome RNA). The newly synthesized full length +RNA can serve as template for further genomic -RNA, as mRNAs for viral protein production or as genomic RNA for packaging into viral progeny, while sub-genome length +RNA strands are used as a transcript for structural and accessory protein synthesis (reviewed in [63, 86]). The ssRNA template is expected to thread its way up to the active site, where incorporation of matching NTPs into the nascent strand occurs, fed through a separate tunnel [65]. At the RdRp active site, incoming NTPs base pair with the RNA template strand, while the 2' and 3' hydroxyl groups form hydrogen bonds with the polymerase [65]. During elongation, a helical double-stranded (ds) RNA product is formed consisting of the RNA template bound to the newly synthesized RNA strand, as depicted in Fig. 1 [66, 69].

The acquisition of a mini-RTC structure by expression of recombinant proteins and assembly of a nsp12-nsp7-nsp8_2-nsp13 complex allowed to better define the interactions between these replicase subunits (Fig. 2A). Nsp13 is the helicase, capable of unwinding RNA in a 5'-to-3' direction [87, 88] and presumably able to clear RNA secondary structures (or RNA-binding proteins), resulting in a single-stranded template that can be used for RNA synthesis. The nsp12-nsp7-nsp8_2-nsp13 complex displayed an increased *in vitro* helicase velocity compared with nsp13 alone [72]. The majority of complexes obtained *in vitro* were formed by two subunits of nsp13, each interacting with the N-terminal region of a nsp8 subunit. The nsp12-nsp7-nsp8_2-nsp13_2 complex showed better resolution and stability than the one formed with only a single nsp13 subunit [72, 73], which suggests that this six multi-part complex is most likely to prevail. The fact that the nsp12-RdRp and nsp13-helicase would translocate in opposite directions, 3'-to-5' and 5'-to-3', respectively, during RNA chain elongation presents a conundrum [73]. In order to explain how the two enzymes may work together, it was proposed that the polymerase may be pushed backwards on the template strand, a mechanism called backtracking. This mechanism has been well-characterized, mainly in DNA-dependent RNA polymerases (DdRp [89]), and has been observed for other viral RdRps [90, 91]. During backtracking, the RdRp would be temporarily inactive (i.e., not performing NTP incorporation) and the template RNA would be held by the helicase, while the product RNA (ssRNA) would be extruding from the RdRp through a secondary channel (Fig. 2B), resembling the one in DdRps that can accommodate single-stranded nucleic acids [73]. This mechanism was demonstrated to help rescue of stalled elongation complexes and removal of misincorporated nucleotides in bacterial and eukaryotic multi-part RNA polymerase complexes (reviewed in [92]). As well, in bacteria, backtracking can be induced by helicases [93], suggesting that the hypothesized backtracking process for nsp12-nsp7-nsp8_2-nsp13 is widespread among different RNA polymerase complexes. In the case of CoVs, a backtracking

mechanism could facilitate the process of template switching during synthesis of sgRNA [94], and/or it could make the 3'-end of the nascent strand accessible for proofreading [73, 95].

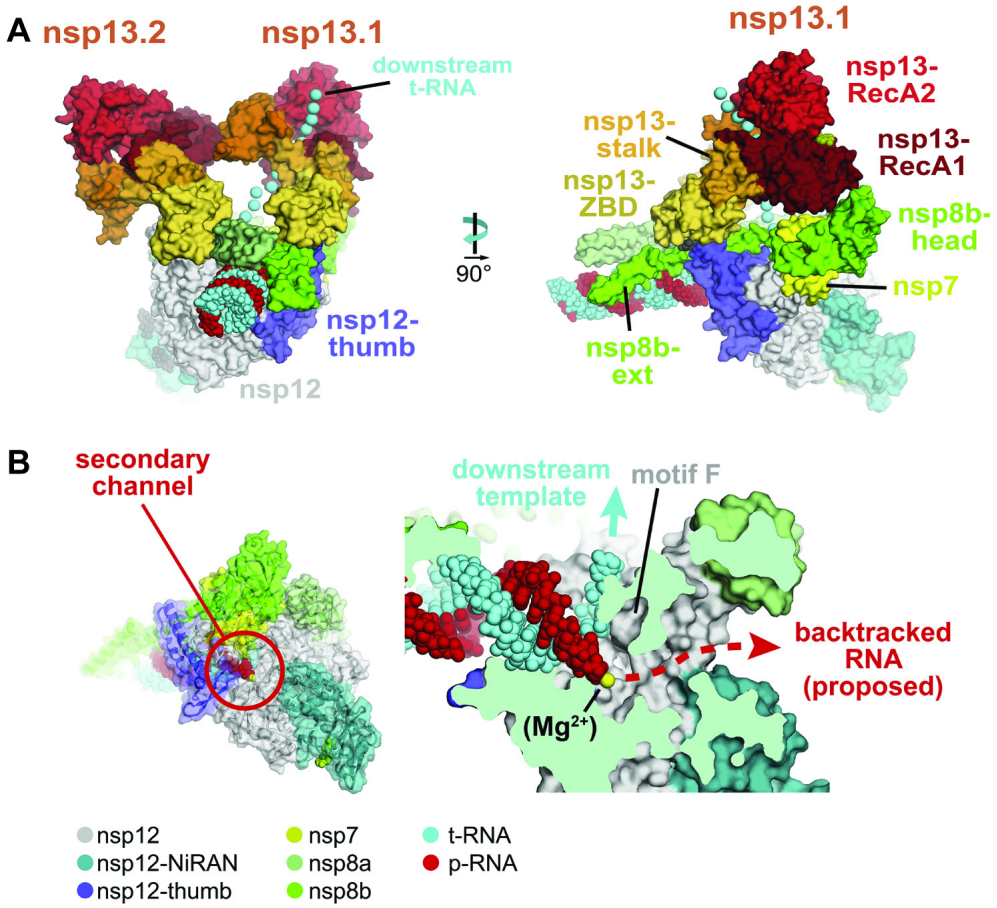

Figure 2- (A)Schematic illustration of the structure of a SARS-CoV-2 mini-RTC composed of nsp7, nsp8, nsp12 and nsp13 subunits. Two copies of nsp13 (nsp13.1 and nsp13.2) form a complex with nsp8 (extension of nsp8b) and nsp12 (thumb). The nsp13-helicase unwinds downstream dsRNA. (B) Localization of the secondary channel in the RdRp structure. The color coding in panel A and B are consistent.

During RNA synthesis, the CoV RdRp can erroneously incorporate mismatching NTPs, which are thought to be detected and excised by a 'proofreading' mechanism in which the central player is the nsp14 exoribonuclease (ExoN) [96], as described in more detail in **chapters III** and **IV**. In this manner, nsp14-ExoN is thought to decrease the CoV mutation rate. For maximal ExoN activity, nsp14 needs to form a complex with nsp10, which results in a conformational change that positions the ExoN catalytic residues closer together and enhances the enzymatic

activity [97]. The nsp10/nsp14 complex is thought to interact with the nsp12-nsp8$_2$-nsp7-nsp13 multimer in order to access a mismatched nucleotide and excise it from the 3' end of the nascent strand using its 3'-to-5' exonuclease activity [77, 96, 97]. Based on biochemical assays, this ExoN activity can use both ssRNA or RNA duplexes as a substrate [97, 98], although the latter is too large to fit in the RdRp secondary channel (Fig. 2B). Correction of dsRNA mismatches was only demonstrated *in vitro* by performing biochemical assays using nsp12-nsp7-nsp8 mixed with nsp10/nsp14 [96]. Supposedly, in this mechanism, nsp10/nsp14 complex would operate near the exit channel of the RdRp on the synthesized dsRNA (composed of the RNA template and RNA product). In this manner, the sequential recognition of mismatches, their excision and the incorporation of the correct NTP should be possible in a coordinated fashion. However, the structural analysis of the mini RTC (Fig. 2A; nsp12-nsp7-nsp8$_2$-nsp13($_2$)) revealed that the RdRp active site is too narrow to accommodate the nsp10/nsp14 complex [73], which suggests that error correction upon backtracking would be more feasible. Based on these structural considerations, the proposed mechanism is that nsp10/nsp14 binds at the mouth of the RdRp secondary channel (Fig. 2B), where it would encounter the 3' end of the nascent RNA strand. Then, the nsp10/nsp14 would have to hydrolyze this RNA, starting at its 3' end, until the mis-incorporated nucleobase is removed. This would require a tight coordination between all RTC subunits (nsp13, nsp12-nsp7-nsp8$_2$, nsp10/nsp14), like a delicate dance with back and forward steps, in order to guarantee the synthesis of a continuous and correct RNA strand. According to this model, the helicase would have a role in promoting the replication fidelity by directing the RNA towards the nsp14-ExoN active site. To date, an nsp10/nsp14 structure has been obtained only for SARS-CoV [96, 99] whereas a structure for the nsp14-ExoN domain in complex with nsp10 was recently reported for SARS-CoV-2 [100]. Due to stability and solubility issues, a structure of nsp12-nsp7-nsp8$_2$ or nsp12-nsp7-nsp8$_2$-nsp13 in complex with nsp10/nsp14 has not been acquired thus far, leaving the question unanswered whether the assembly of this multi-protein complex is possible and which motif or domain of nsp12 would interact with the nsp14.

The fact that some nucleoside/nucleotide analogues are capable of stalling RNA elongation whereas others fail due to the ExoN proofreading function [101], emphasizes how critical it is to better understand the CoV RNA replication/transcription mechanism. Future studies will be needed to clarify how the nsp10/nsp14 complex interacts with the RTC and how post-transcriptional modifications involving these and other replicase subunits are performed. Still, most of the hypotheses described above remain to be investigated by enzymatic assays and extrapolated to the context of the RTC in the living CoV-infected cell.

Messing up the code: Nucleoside/ nucleotide analogues

To date, one of the most extensively explored classes of antiviral drugs are nucleoside analogues, synthetic derivatives of natural purines and pyrimidines in which the sugar moiety and/or the heterocyclic ring is altered. These compounds can be formulated as nucleoside precursors, prodrugs or nucleotides (mono-, di- or triphosphorylated). Once administered, with the exception of NTPs, they will (presumably) be taken up by the cell and metabolized by host kinases to their active NTP form. Then, these compounds should be recognized by the viral replication/transcription machinery and incorporated into a nascent RNA chain. Inhibition can be achieved by one or more mechanisms including premature termination of RNA elongation by RdRp stalling, depletion of cellular NTP pools, or induction of mismatches that lead to accumulation of (deleterious) mutations [101, 102], also referred to as 'lethal mutagenesis' [103].

Some examples of nucleoside analogues previously reported to have antiviral activity against CoVs are listed in Table 1. In general, studies in which CoVs were cultured in the presence of nucleoside analogues demonstrated a relatively high barrier to antiviral drug resistance [34, 39, 104, 105]. To acquire resistance to nucleoside analogues, changes should be acquired in or near the RdRp's active site, which might interfere with its enzymatic activity and consequently viral fitness. For example, the two resistance mutations identified in the RdRp domain of MHV after passaging in the presence of remdesivir conferred partial resistance to the compound and reduced the fitness of MHV or SARS-CoV mutants in competition studies with wt virus [34]. Interestingly, the same mutations alone or together increased CoV sensitivity to B-D-N4-hydroxycytidine (NHC) [42]. Based on the overall conservation of the NTP binding site across CoV subgenera and in other viruses [106], compounds targeting viral RdRps can act as broad-spectrum inhibitors and that is why in the case of a newly emerging pathogen they usually are one of the first drug classes tested in drug re-purposing programs.

In the case of CoVs, the presence of a proofreading enzyme, nsp14-ExoN, may provide resistance or elevated tolerance to antiviral nucleoside analogues. Therefore, in order to efficiently inhibit CoVs, compounds should be incorporated by the RdRp while evading ExoN-mediated excision, or at least be incorporated at a much higher rate than ExoN can use to remove them. NHC displays a high resistance to ExoN excision, introducing an intolerable number of mutations in the viral genome at low micromolar concentrations in cell culture [39, 42]. This suggests that this nucleoside analogue is a mutagenic agent promoting lethal mutagenesis. Structural studies demonstrated that the incorporation of nucleoside analogues like remdesivir results in termination of RNA elongation after a limited chain extension (non-obligate termination; Table 1). In this case, the incorporated nucleotide analogue is buried in

the RdRp's active center and prevents the translocation of RNA (stalling) after the incorporation of 3 additional NTPs. This is explained by remdesivir's chemical structure, in which the cyano-group clashes with the RdRp's thumb domain [107, 108]. This may complicate access by the nsp14/nsp10 complex and could allow remdesivir to escape from ExoN-mediated excision [108]. Moreover, this may explain why remdesivir is less efficient in inhibiting wt virus than ExoN-knockout mutants [34].

In order to increase the efficacy of nucleoside analogues to inhibit CoVs, one could target both the RdRp and the ExoN activity using combination therapy. As demonstrated in cell culture, inactivation of ExoN increases the sensitivity of SARS-CoV and MHV to nucleoside analogues, or abrogates the production of viral progeny of other CoVs, including MERS-CoV and SARS-CoV-2 (**chapter IV)**. This suggests that targeting both the RdRp and ExoN is may be a good antiviral strategy. To date, screening of marketed small molecules identified two compounds (ATA and patulin) that efficiently inhibit ExoN activity in biochemical assays at low-micromolar concentrations (calculated EC_{50} values of 1.25 and 20 µM, respectively). However, a high cytotoxicity and poor inhibitory activity were demonstrated in Vero E6 cells infected with SARS-CoV-2 [109]. The nsp14-ExoN of CoVs and other nidovirus members ([110] and **chapter III**) as well as the Ebolavirus nucleoprotein exonuclease [111, 112] belong to the DEDDh family of exonucleases. Therefore, analysis of compounds that target enzymes belonging to this family can lead to the identification of broad-spectrum inhibitors or help to understand the mode of action of prospective molecules. Structure modelling of another DEDDh exonuclease (CRN-4) with inhibitors like ATA, MES and pontacyl violet 6R elucidated the binding at the compound-enzyme interface and identified the residues involved in this interaction [113]. This suggests that targeting nsp14-ExoN activity directly may be possible, although , on the down side, the lack of a pocket in this domain main will remain a major challenge [114].

Table 1- List of nucleoside analogues with described CoVs antiviral activity in infected cells, animal experiments or clinical trials

Compound Name	Derivatives	Analogue of	Proposed mode of action against CoVs	Antiviral effect*	Ref.
Acyclovir (fleximer)	Ganciclovir CMPD3	Guanosine		HIV; VZV; HSV; MERS-CoV; HCoV-NL63	[3, 115, 116]
AT-527	AT-511, AT-9010	Guanosine	Stops elongation of RNA chain	SARS-CoV-2, HCV	[85]
BCX4430 (Galidesivir)		Adenosine	Stops elongation of RNA chain Binds to RdRp	MERS-CoV, SARS-CoV, SARS-CoV-2, influenza	[117, 118]
B-D-N4-hydroxycytidine (NHC)	MK-4482 (Molnupiravir)/ EIDD-2801	Cytidine	Lethal mutagenesis Resistant to ExoN activity	MERS-CoV, SARS-CoV, SARS-CoV-2, HCoV-NL63, HcoV-OC43, VEEV, CHIKV	[39, 42, 119, 120]
Gemcitabine hydrochloride		Cytidine	Stops elongation of RNA chain	MERS-CoV, SARS-CoV, SARS-CoV-2, ZIKV, Influenza	[121-123]
GS-5734 (Remdesivir)	GS-441524	Adenosine	Stops elongation of RNA chain Competition with ATP for RdRp binding Mutagenic potential	MERS-CoV, SARS-CoV, SARS-CoV-2, HcoV-OC43, EBOV	[34, 35]
Mizoribine		Imidazole		SARS-CoV	[124]
Ribavirin		Guanosine	Reduction of NTP pools	MERS-CoV, SARS-CoV, RSV, HCV	[104, 125]
Sofosbuvir		Uridine	Stops elongation of RNA chain	HCV	[118, 126]
T-705 (Favipiravir)	T-1105	Guanosine	Stops elongation of RNA chain Lethal mutagenesis	SARS-CoV-2, Influenza, WNV, HCV	[106, 127-129]
Tenofovir		Adenosine		HIV, HBV	[130]
5-fluorouracil		Uridine	Lethal mutagenesis	SARS-CoV	[104]
6-Azauridine		Uridine		HcoV-NL63	[131]

VEEV, Venezuelan equine encephalitis virus; HBV, hepatitis B virus; VZV, Varicella zoster virus; Ref., references;

Look at the other side of nsp14: N7-MTase as a target

Coronavirus nsp14 is a bi-functional protein known to perform two activities *in vitro*: excision of nucleotides in a 3'-to-5' direction and methylation of the N7 position of the CoV mRNA cap structure, as described in **chapter V**. Presumably, CoVs are dependent on this cap-structure for translation of their genomes into proteins by cellular ribosomes. This 5' cap structure avoids viral mRNA to be recognized as "non-self" by multiple innate immune sensors and protects it from degradation by host 5'-to-3' exoribonucleases. Characterization of N7-MTase mutants revealed that this protein is important for the viability of different CoVs (**chapter V**) and for replication in cell culture [132]. Thus, the CoV N7-MTase may constitute an attractive target for antiviral drug development.

A comparison of allosteric and catalytic pockets of all viral proteins across different α- and β-CoVs revealed that the nsp14-N7MTase is highly conserved, presenting 60% conservation at the amino acid level and (for example) 100% identity between SARS-CoV and SARS-CoV-2 [114]. In addition, the nsp14-N7-MTase domain presents a unique non-Rossmann fold that distinguishes it from the majority of known cellular and viral MTases. To date, the development of different types of assays for characterization and evaluation of N7-MTase activity has been reported, leading to the identification of potential inhibitors targeting this domain, most of them *in vitro* [133-139]. Thus far, three different approaches have been used for developing nsp14-N7-MTase inhibitors. One strategy is the design of compounds directly targeting the N7-MTase, based on the structure of its catalytic pocket, like aurintricarboxylic acid (ATA) and sinefugin. Another approach is to design analogues of SAM, the main methyl donor used by viral and cellular MTases. Consequently, these compounds will act as competitors of MTase SAM-dependent like SAH, which binds more strongly to CoVs MTases than SAM itself [135]. Alternatively, development of inhibitors that can interfere with the production of SAM by targeting molecules involved in its metabolism may reduce its intracellular levels and block (indirectly) MTase activity, like S-adenosylhomocysteine (SAH) or adenosine analogues such as aristeromycin (**chapter VI**). Challenges for the development of specific viral MTase inhibitors include the lack of specificity, high cytotoxicity and poor bio-availability which may have contributed to the poor correlation between results from structural, biochemical and cell-based screening of N7-MTase compounds. This suggests that improved drug design and more-translatable models that can corroborate enzymatic assays with infected cell-based screenings need to be pursued.

Nsp14: the constant gardener promoting viral fitness and fidelity?

The replication of +RNA virus genomes is generally characterized by high error rates, large viral progeny sizes and short replication times [101]. Consequently, a cloud of closely related viral genomes is generated with variable degrees of fitness, the so-called quasispecies population. The accumulation of mutations can result in loss of fitness or error catastrophe, but on the other hand the adaptation of RNA virus population to changing circumstances depends on the generation and selection of beneficial mutations [103]. Distribution of these mutations is unevenly throughout the genome and different factors may contribute to the frequency of mutations derived from low-fidelity RdRps.

The mutation rate is correlated with RdRp speed (rate at which the polymerase incorporates NTPs into the RNA chain during synthesis) and accuracy (selection of the correct NTP according to the Watson-Crick base pair geometry). For CoVs, RdRp speed *in vitro* using the nsp12-nsp7-nsp8$_2$ complex was estimated to range from 10 to 100 nt per second [127]. Compared to the RdRps from e.g. poliovirus, which displays a similar structural organization, the SARS-CoV-2 RdRp is rather fast, presenting a 3 to 10 times higher speed of NTP incorporation [106, 140, 141]. Taking into account that CoV genomes are around 30 kb long instead of the more common 7-15 kb seen in other RNA viruses, it can be presumed that the CoV RdRp is faster. However, *in vitro* data revealed that if this is the case, this appears to come at a cost: an increased level of mismatch incorporation compared to e.g. DENV and other RNA viruses presenting a shorter genome length [96, 142]. The generally restricted genome size of +RNA viruses, below 15 kb in length, is presumably associated with the lack of proofreading mechanisms. This constraint has been linked to having low genetic complexity [143, 144]. Replication fidelity, genome size and complexity are trapped in a triangular relation known as the Eigen paradox [145], which tries to explain the evolutionary state of +RNA genome sizes. In contrast, most nidoviruses, including CoVs, possess a genome larger than 20 kb. The identification of an ExoN domain in all these large-genome nidoviruses and the attribution of a proofreading function to this protein may explain how they balance their RdRp fidelity and genome size [143, 146, 147]. With some exceptions [110], the ExoN enzyme has been mainly characterized for CoVs, as described in **chapter III**.

For two CoVs, the impact of ExoN knock-out mutations on viral RNA synthesis has provided direct experimental evidence that this enzyme boosts replication fidelity. ExoN-knockout mutants of SARS-CoV and MHV displayed an increased mutation frequency compared to the corresponding wt virus [148-150].In contrast, the equivalent mutants of five other CoVs proved to be non-viable: MERS-CoV, SARS-CoV-2 (described in **chapter IV**), HCoV-229E [98], TGEV [151] and IBV (personal communication by E. Bickerton *et al.*, described in [152]). This

extreme phenotypic difference between SARS-CoV and MHV on the one hand and different α-, β- and γ-CoVs on the other hand suggests that the first two viruses may somehow be exceptions to the rule. Although both the MHV and SARS-CoV ExoN knockouts are somewhat crippled in their replication, they can apparently tolerate the impact of the ExoN knockout on virus replication, fidelity and fitness for many passages.

In-depth analysis of the genome of passaged MHV-ExoN-knockout mutants revealed that acquisition of compensatory mutations in different regions of the genome, including nsp12, is critical for viral fitness in the absence of a functional ExoN [153]. Exchanging nsp12 and nsp14 of this MHV-ExoN knockout mutant (including compensatory mutations) for their wt counterparts was detrimental to viral replication and competitive fitness. As reversion of the mutations in the MHV-ExoN knockout mutant was not observed, this suggests that the mutations acquired during passaging adapted the virus to overcome or tolerate the limitations on viral RNA synthesis and replication posed by the selective pressure of having a non-functional or partially functional ExoN. Analysis of intermediate revertants of this MHV ExoN-knockout mutant (D89A/E91A), obtained by single alanine or conservative substitutions of the first two catalytic residues of the DEDDh motif, demonstrated no increased replication or competitive fitness [153]. In addition, it seems that there is no clear benefit for reversion from MHV ExoN-knockout to wt to occur, which might explain why this was not observed, not even after 250 passages [154]. While for MERS-CoV and HCoV-229E conservative and/or alanine mutations were analyzed for each single residue of the DEEDh motif (**chapter IV** and [98]), for SARS-CoV and MHV only substitutions of the first two catalytic residues were evaluated. This leaves the question if replacement of other residues in MHV and SARS-CoV would lead to the same phenotypic profile. Overall, these observations suggest that nsp14 or nsp14-ExoN plays a more direct and basic role that is critical for RNA synthesis and not only correlated with promoting the fidelity of virus replication.

Recently, CoV ExoN was also proposed to play a role in RNA recombination [155]. In viruses with a non-segmented RNA genome, recombination can occur between two distinct RNA molecules (inter-molecular) or within the same RNA molecule (intra-molecular). Two mechanisms have been hypothesized: recombination by template switching and recombination by non-replicative breakage and rejoining [156]. As part of the discontinuous minus-strand RNA synthesis that CoVs employ during the production of their subgenomic RNAs, a form of recombination occurs for which base pairing between complementary TRS sequences (minus body TRS to plus leader TRS) is a crucial determinant. In addition, similar RdRp template switching behavior may lead to the generation of defective genomes with large deletions, which can be replicated *in* trans by the full-length helper virus if they contain

the signals for initiation of RNA synthesis. Therefore, such defective genomes may interfere with virus replication and if they are packaged this yields defective interfering particles, which may be infectious and/or strong inducers of cellular immune responses [157, 158]. Both these processes, production of sgRNAs and of defective genomes, affect the replication of virus. In order to analyze the occurrence of recombination in CoVs, NGS and full-length direct RNA sequencing was performed, which allows correlation of mutations/recombination occurring in the same RNA strand [155, 159, 160]. In these studies, the occurrence of recombination was identified by the formation of junctions, derived from RdRp template jumping or switching between two non-contiguous sequences. From the recombination events, defective viral particles (containing deletions of genomic sequence while retaining 5' and 3' untranslated regions), canonical sgRNAs and alternative sgRNAs were generated. When analyzing the genetic profile of different β-CoVs, MHV, MERS-CoV and SARS-CoV-2, similarities were observed in terms of the position of junctions in the genome, frequency of junctions (proportion of junctions in a population of genomes) and nucleotide composition of junctions. Strikingly, a higher recombination frequency was observed in SARS-CoV-2 samples when compared to MERS-CoV and MHV, which might promote viral sequence variation and adaptation to selective pressures [155]. In this study, a role for CoV ExoN in recombination was proposed based on the reduced recombination frequency observed for an MHV ExoN knockout mutant [155]. Mainly, an increased abundance of canonical and alternative sgRNA was detected in both intracellular and supernatant RNA of MHV-wt. Already, when MHV ExoN-knockout mutants were characterized previously, a reduced abundance of sgRNA 2 was noticed [148]. Moreover, for non-viable HCoV-229E ExoN knockout mutants an increase of the relative amount of sgRNA 4 to 6 and the appearance of an aberrant sized sgRNA 3 was previously reported [98], suggesting that ExoN inactivation can impact sgRNA production. As a proof-reading enzyme ExoN must interact with the RTC and may interfere with some of its other activities e.g. elongation and backtracking ([94, 161] see above). Thus, ExoN may affect RdRp processivity, as proposed in mechanistic models based on the acquired enzyme structures and *in vitro* experiments [161, 162]. Consequently, this might indirectly influence viral recombination and alter the production of defective viral genomes, canonical or non-canonical sgRNAs which subsequently may affect viral progeny viability and infectivity.

THE NEXT CHAPTER – SOME POINTS OF REFLECTION

The on-going COVID-19 crisis revealed the limitations of our knowledge about CoVs and the lack of preparedness for this scale of events. Thus, in the wake of the SARS-CoV-2 pandemic, efforts to understand how CoVs replicate and interact with their host were increased. Looking

at the latest discoveries in CoV molecular biology, obtained using e.g. refined structural and biochemical techniques, it seems only natural that a synergetic translation of *in vitro* models to the context of the infected cell will be explored. Mechanistic studies that unraveled interactions between different replicase subunits demonstrated that the RTC revolves mainly around a subset of subunits: nsp12-nsp8$_2$-nsp7, nsp13, nsp10/nsp14, nsp16/nsp10 and nsp9. Dynamic interactions between these RTC subunits can be (temporarily) established and coordinate all mechanisms around RNA synthesis and post-transcriptional modifications. The identification of potential structural features that mediate these interactions and genetic markers that define functional domains is important to understand how sequential activities are performed. In addition, this can help to appreciate evolutionary pathways. The mechanisms underlying several processes carried out by the CoV RTC remain to be elucidated, such as initiation of RNA synthesis, RdRp template switching during sgRNA synthesis, proof-reading and viral mRNA capping. Taking into consideration the importance of these mechanisms for viral replication (**chapter IV** and **V**), this might define promising new targets for antiviral drug development.

Trying to create broad(er)-spectrum prophylaxis and therapy remains a major goal to solve the current pandemic and prepare for future emerging coronaviruses. The genetic plasticity of +RNA virus genomes presents a challenge for controlling virus spread, retaining vaccine efficacy and the development of efficient antivirals, as it drives the rapid development of escape variants while preserving overall viral fitness. Already, during recent months, a large number of SARS-CoV-2 'variants' have attracted attention, including B.1.1.7 (also known as the British variant), B.1.351 (South African variant), B.1.1.28.P1 (Brazilian variant) and most recently B.1.617 (Indian variant). Many of these are being monitored and characterized to understand their clinical and epidemiological relevance. In general, besides having several poorly understood mutations elsewhere in the genome, these variants carry spike protein mutations that have been flagged for (presumably) enhancing virion infectivity and/or potentially affecting vaccine efficacy and neutralization by therapeutic monoclonal antibodies [163-165]. This emphasizes the urgent need to invest in preparedness through surveillance of circulating viruses, implementation of broad-spectrum antiviral drugs and other therapies, and development of adaptable platform for vaccine production that can be easily deployed to address new SARS-CoV-2 variants (if necessary), other CoVs or at large other viruses. In sum, *an ounce of prevention is worth a pound of cure.*

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