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## **The quest for broad-spectrum coronavirus inhibitors**

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### **Citation**

Lima Leite Ogando, N. S. (2021, October 12). *The quest for broad-spectrum coronavirus inhibitors*. Retrieved from <https://hdl.handle.net/1887/3217007>

Version: Publisher's Version

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**Note:** To cite this publication please use the final published version (if applicable).

## ENGLISH SUMMARY

Up to the present time, seven coronavirus (CoVs) that can infect humans have been identified: four endemic human CoVs (229E, NL63, OC43 and HKU1), responsible for common cold symptoms, and three zoonotic CoVs (SARS-CoV, MERS-CoV and SARS-CoV-2) that can cause severe disease. The latter viruses emerged in the last 20 years. The potential for zoonotic transmission and global spread demonstrated by the pandemic SARS-CoV-2, and its burden on public health, have emphasized the critical need to develop highly efficacious strategies for prophylaxis and therapy of infections with CoVs at large. In **chapter I**, a timeline of CoV discovery and a broader overview of their biology, replication cycle, and pathogenesis are presented. Furthermore, some findings regarding vaccine development and antiviral and antibody therapies are highlighted.

The research described in this PhD thesis was largely dedicated to the quest for broad-spectrum CoV inhibitors. In the beginning of this project, compounds were tested mainly in cells infected with MERS-CoV, whereas after February 2020 the evaluation of antiviral activity against SARS-CoV-2 was also included. **Chapter II** describes the characterization of some of the first SARS-CoV-2 isolates and the adaptation of *in-house* phenotypic screening assays to determine the sensitivity of SARS-CoV-2 to different inhibitors such as remdesivir, alisporivir, chloroquine, and pegylated interferon alpha. This type of assays was used in **chapters VI** and **VII** to evaluate the inhibitory effect of 6',6'-difluoro-aristeromycin (DFA) and FDA-approved compounds, respectively. In **chapter VI**, DFA, a small molecule originally designed to target S-adenosylhomocysteine (SAH) hydrolase was characterized by phenotypic studies and genotyping of drug-resistant mutants. This compound can strongly inhibit MERS-CoV replication and completely abrogate the production of viral progeny at low-micromolar concentrations. Based on our results, we hypothesize that this small molecule may affect intracellular levels of the methyl donor S-adenosylmethionine, which is used by the two CoV methyltransferases involved in the processing of the cap that is present at the 5'-end of all viral mRNAs. The cap structure is important for translation of viral mRNAs by host ribosomes, protection of these RNAs from exoribonuclease hydrolase activity, and escape from immune sensors. In **chapter VII**, calcineurin inhibitors (CNI) that possess antiviral activity against RNA viruses were tested against SARS-CoV-2. Cyclosporine A (CsA, a known broad-spectrum inhibitor of human and zoonotic CoVs) and voclosporin (VCS, a novel CNI structurally similar to CsA), were demonstrated to have antiviral activity against SARS-CoV-2. In particular, VCS reduced the SARS-CoV-2 load in lung cells at concentrations that are safe in humans and at lower concentrations than CsA and tacrolimus (TAC). The efficacy of approved vaccines is yet uncertain in kidney transplant recipients (KTRs), in which immunosuppressive and non-

immunosuppressive therapies like CsA and TAC are commonly administered to prevent rejection of the transplant. Based on our *in vitro* experimental data, we argue that there may be a potential benefit of the use of VCS to treat COVID-19 in KTRs undergoing an immunosuppressive regimen. These data warranted the further clinical investigation of VCS in SARS-CoV-2-infected KTRs, which is currently in progress.

The development of antiviral therapies requires a detailed understanding of CoV replication and its interplay with host cells. SARS-CoV and SARS-CoV-2 belong to the same CoV species, showing a limited genetic distance and essentially the same genome organization. In order to understand potential differences between SARS-CoV and SARS-CoV-2 replication in infected cells, a comparison of various replication features was performed, including RNA synthesis, the production of viral progeny, and cytopathology upon infection (using electron microscopy and immunolabelling), which is described in **chapter II**. One important difference between these two viruses is the presence of a “furin-like cleavage site” in the region connecting the S1 and S2 domains of the SARS-CoV-2 Spike (S) protein. Adaptative mutations in this S region evolved upon passaging of SARS-CoV-2 in Vero E6 cells (the most common cell line used), resulting in phenotypic changes. According to other studies, the change or loss of this site can potentially affect infection efficiency in other (more relevant) cell lines, e.g., lung cells. Over the past three decades, CoV replicase proteins have been characterized using a combination of bioinformatics, biochemistry, structural biology, and (reverse) genetics. **Chapters III to V** describe the in-depth characterization of CoV nsp14, one of the enzymatic components of the replication and transcription complex (RTC), which provided evidence for its importance for virus viability and fitness, while also establishing that nsp14 might be a good target for drug design. This bifunctional protein contains 3'-to-5' exoribonuclease (ExoN) and guanine-N7-methyltransferase (N7-MTase) domains that are described in **chapters IV** and **V**, respectively. Supported by the increasing availability of structural information for SARS-CoV nsp14, key residues of both nsp14 domains were identified and their importance was probed by site-directed mutagenesis. Phenotypes of engineered mutant CoVs, launched from cloned cDNA templates, were analyzed, as well as enzymatic activities of corresponding recombinant proteins using *in vitro* assays. The data in **Chapter IV** demonstrate that CoV nsp14 ExoN activity is critical for primary viral RNA synthesis and apparently has an additional role in viral replication, besides mediating error-correction (proofreading) of mismatches incorporated by the viral polymerase during genome replication. It was found that both MERS-CoV and SARS-CoV-2 cannot tolerate an ExoN knockout mutation, in contrast to what was previously reported for MHV and SARS-CoV. In **Chapter V**, evidence is presented that also the nsp14 N7-MTase domain/activity is important for *Betacoronavirus* viability and that there might be

structural differences between MHV, SARS-CoV, SARS-CoV-2, and MERS-CoV, despite the strong conservation of the N7-MTase amino acid sequence. Three substitutions led to the same phenotypic profile and they define key residues of the N7-MTase catalytic pocket that can be targeted to design inhibitors with a potential pan-coronaviral activity spectrum. Mechanistic hypotheses on how nsp14 interacts with other subunits of the RTC during RNA synthesis and post-transcriptional processes are described in **chapter VIII**. Additionally, in this concluding chapter, the history of unsuccessful CoV-targeting antivirals is briefly summarized, together with possible new approaches in antiviral research. Lastly, some prospects for future research are outlined.