

UNIVERSITY OF PÉCS

Biological and Sportbiological Doctoral School

**Antiviral activity evaluation of pharmaceutical drugs, newly
synthesised compounds, and fragments against SARS-CoV-2 *in
vitro***

PhD Thesis

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PÉCS, 2023.

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

~ Marie Curie ~

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Abbreviations

+ssRNA–positive sense, single-stranded ribonucleic acid

Bcl-2-B-cell lymphoma 2

CC₅₀-50 % cytotoxic concentrations

COVID-19-coronavirus disease 2019

CoVs-coronaviruses

CPE-cytopathogenic effect

CQ-hydroxychloroquine

ddPCR-Droplet-digital polymerase chain reaction

DENV-dengue virus

E-envelope

e.g.- exempli gratia (lat.) for example

EBOV-ebola virus

EMA-European Medicines Agency

et al.-et alii/et aliae/et alia (lat.) and others

etc.-et cetera (lat.) and so forth, and so on

EUA-Emergency Use Authorization

FDA-Food and Drug Administration (USA)

hACE2-human angiotensin-converting enzyme-2

HBV-hepatitis B

HCQ-hydroxychloroquine sulfate

HCV-hepatitis C virus

HIV-human immunodeficiency virus

HSV-herpes simplex virus

IAV-influenza virus A

IBV-influenza virus B

IC₅₀-50 % inhibitory concentration

ICU-intensive care unit

IFN-interferon

IL-interleukin

IRF3-interferon regulatory factor 3

ISG-interferon-stimulated gene

LASV-lassa virus

M-membrane

MERS-CoV-Middle East respiratory syndrome coronavirus

MOI-multiplicity of infection

mRNA-messenger RNA

N-nucleoprotein

NiV-nipah virus

nsp-non-structural protein

ORF-open reading frame

PARP-Poly (ADP-ribose) polymerase

PV-poliovirus

R&D-research and development

RdRp-RNA-dependent RNA polymerase

RSV-respiratory syncytial virus

RTC-replication and transcription complex

S-spike

SARS-CoV-severe acute respiratory
syndrome coronavirus

SARS-CoV-2-severe acute respiratory
syndrome coronavirus 2

SINV-sindbis virus

TBEV-tick-borne encephalitis

TMPRSS2-transmembrane serine protease
2

VACV-vaccinia virus

VOC-variant of concern

WHO-World Health Organization

WNV-West Nile virus

YFV-yellow fever virus

ZIKV-zika virus

1. Introduction

1.1. The newly emerged coronavirus, the SARS-CoV-2

In December 2019, there was a high incidence of hospitalisation with severe pneumonia in Wuhan (Hubei province, China). It was later confirmed that the patients were infected with a newly emerged zoonotic agent, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Most of the early infections were clustered in central Wuhan, where the Huanan Wholesale Seafood Market is also located. Additionally, the seroprevalence of SARS-CoV-2 was also the highest in the district around the market. In the Huanan Market, besides seafood and poultry, live, wild-captured or farmed mammalian species such as raccoon dogs (*Nyctereutes procyonoides*), hog badgers (*Arctonyx albogularis*) and red fox (*Vulpes vulpes*) were also sold. SARS-CoV-2 RNA was detected in a few environmental samples from sewerages and stalls where live animals were sold. However, there are not any reports about SARS-CoV-2 tests from animals sold in the market [1]. Several groups have studied how SARS-CoV-2 could emerge. Phylogenetically, it belongs to the *Coronaviridae* virus family and clusters with SARS-CoV and other coronaviruses (CoVs), detected in bats (for example RaTG13, RmYN02) and pangolins (originating, e.g., Guangdong and Guangxi province) so far and forming together the Sarbecovirus subgenus within the Betacoronavirus genus. The SARS-CoV-2 genome is similar to SARS-CoV in 79 % and shares 50 % genome sequence identity with middle east respiratory syndrome coronavirus (MERS-CoV). Its structural-, and non-structural protein-coding genes share 90 % and more than 85 % amino acid sequence identity with SARS-CoV, respectively. Bat-derived CoVs, the RaTG13 from a *Rhinolophus affinis* bat and the RmYN02, from a *Rhinolophus malayanus* bat, are ~96 % and ~93 % identical to SARS-CoV-2, respectively. The sequences from pangolins from Guangdong province show 92.4 % sequence similarity, while the sequences from Guangxi show 85.5 % sequence similarity with SARS-CoV-2. The most notable distinguishing features of SARS-CoV-2 are within the S protein. Five of the six amino acid residues within the receptor binding domain (RBD) differ between SARS-CoV-2 and SARS-CoV. In the junction of the S1 and S2 subunits of the Spike, there is a polybasic cleavage site (RRAR), and proline is also inserted at the beginning of this sequence (PRRAR). This site enables more effective cleavage by the host furin and other proteases. Such a cleavage site is not observed in other Sarbecoviruses except one with a similar cleavage site (P-AAR) in RmYn02. The addition of proline helps establish O-linked

glycosylation around the cleavage site. Presumably, it shields the epitopes, thus helping immunoevasion [2,3]. Due to technical advancements (next-generation sequencing), extensive monitoring of the acquired mutations of the circulating virus was performed worldwide. This led to the rapid identification of newly evolved virus variants. The emerging variants outcompeted the previous variants and became dominant globally. A substitution within the spike was the first outstanding mutation that aided a more efficient transmission (D614G). It was classified within the PANGO lineage B.1. More and more mutated variants have evolved. WHO declared some of these as variants of concern (VOC) requiring more extensive monitoring and functional analyses. VOCs generally became dominant regionally or worldwide rapidly and could escape from the immune response more effectively. B.1 lineage existence faded after the appearance of the Alpha variant (PANGO lineage B.1.1.7). Soon after the detection of the Alpha variant, the Beta variant (B.1.351), the Gamma variant (P.1) and the Delta variant (B.1.617.2) were also detected. Enhanced furin cleavage has been observed in the case of Alpha and Delta variants. The distinguishing feature of Omicron (B.1.1.529) variants is that their entry is somewhat altered and immune escape is more excellent compared to the previously dominant variants. These variants can infect vaccinated or previously infected individuals more effectively. Omicron now has several major lineages (BA.1, BA.2, BA.3, BA.4 and BA.5). From BA.2 several sublineages were derived, such as BA.5, BA.4, and BA.2.75. From BA.5 originated the BQ sublineages, while the BM and XBB sublineages evolved from BA.2.75. The current waves are dominated by the newly risen Omicron variants. Upregulation of viral proteins that inhibit the innate immune system has been described in Alpha and Omicron variants. Clinical manifestations and sensitivity to therapeutics are also diverse within different VOCs. Although the developed therapeutic interventions, immune status (e.g., vaccination) should also be considered when determining the relative severity of infection caused by the different VOCs [4,5].

1.1.1. Viral genome and proteins

The genome of CoVs is generally three times larger than the genomes of other RNA viruses. SARS-CoV-2 has an approximately 30 kb, positive-sense, single-stranded, nonsegmented RNA genome (+ssRNA) that encodes 16 non-structural (nsps) and 4 structural proteins. Two-thirds of the whole genome is the coding region of two polyproteins, the polyproteins 1a and 1ab. The open reading frame 1a (ORF1a) is transcribed into polyprotein 1a (pp1a), while polyprotein 1ab (pp1ab) is produced by a ribosomal frameshift that overreads the STOP codon between ORF1a and ORF1b. These two polyproteins are then further processed. Nsp1-11 are produced by

cleaving the polyprotein 1a, while polyprotein 1ab is further hydrolysed to nsp1-16 (Fig 1) [6,7].

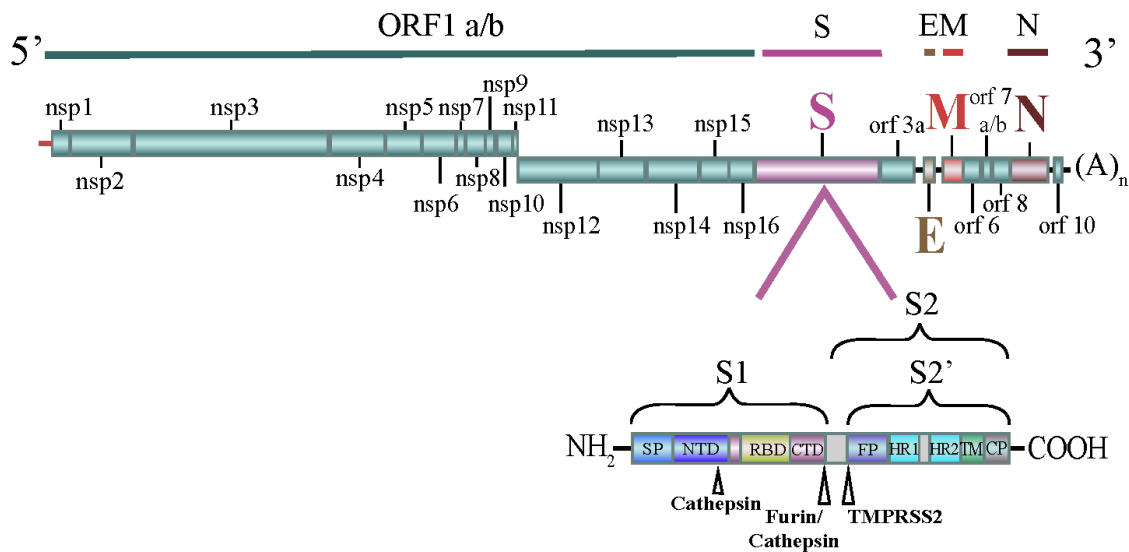


Figure 1. A schematic representation of the SARS-CoV-2 genome: SARS-CoV-2 has a 5'-capped and 3'-polyadenylated RNA genome. It codes two large overlapping open reading frames (ORF1a and ORF1ab) occupying two-thirds of the genome. The remaining 1/3 consists of the genes of the four structural components of the virus (S, M, E, N) and the genes of accessory proteins. ORF 1a and ORF 1ab code non-structural proteins that help the viral genome replication and translation, as some of the enzymatic processes cannot be made by the host cell. In addition, nsp's modulate the host immune system. Spike has two subunits, cleaved by host proteases (cathepsins, furin, TMPRSS2), thus facilitating the viral entry. Abbreviations: ORF-open reading frame, nsp-non-structural protein, S1-receptor binding subunit, S2-membrane fusion subunit, E-envelope, M-membrane, N-nucleoprotein, SP-signal peptide, NTD-N' terminal domain, RBD-receptor binding domain, CTD-C' terminal domain, FP-fusion peptide, HR1 and HR2-heptad-repeat domain, TM-transmembrane domain, CP-cytoplasmic tail [8–11].

Nsp1 inhibits host protein translation by sterically obstructing the ribosomal mRNA entry channel (40S subunit, 43S pre-initiation complex, non-translating 80S subunit), thus weakening the innate immune response, mainly the interferon-stimulated responses, against the invading virus. Interestingly, while the host mRNA translation is paralysed by nsp1, the viral mRNA translation is enhanced. The viral 5' untranslated region (UTR) is probably responsible for this in an unknown manner. In addition to block mRNA binding to the ribosome, nsp1 induces mRNA degradation in the cytosol and interferes with the nuclear export of human mRNA [12–14]. Nsp2 also impedes the innate cellular immune response of the host. It can directly interact with a cellular protein, thus repressing the translation of IFN- β [15]. Nsp3, the papain-like

protease (PLpro), cleaves pp1a and pp1ab to generate nsp1, nsp2, nsp3, and nsp4. Furthermore, one of the macrodomains of PLpro binds to ADP-ribose, which is also believed to hinder the IFN response. It has also been shown that PLpro can cleave the ubiquitin-like modifier ISG15, K48-linked polyubiquitin. Ubiquitin and ubiquitin-like modifier ISG15 attachment to proteins, *e.g.*, interferon regulatory factor 3 (IRF3), is an important step in the innate antiviral and inflammatory response. K48-linked polyubiquitination serves as a signal for proteasomal degradation. ISGylation can interfere with viral assembly and budding. Besides indirectly modulating the IRF3 pathway, PLpro can directly cleave IRF3, one of the key factors of viral infection-triggered type I interferon response. It has also been shown that activation of IFN- β is inhibited in the presence of nsp3. Thus, the immunomodulatory effect of the PLpro protein, besides viral polyprotein cleavage, is also important. Moreover, nsp3 is also a key component of the viral replication and transcription complex (RTC) [11,16–20]. Nsp4 is also part of the RTC. Together with nsp3 and nsp6, it forms double-membrane vesicles (DMVs). Furthermore, in cooperation with ORF9b, nsp4 induces mitochondrial DNA release. It can tether the Bcl-2 homologous antagonist killer (BAK) protein to the mitochondrial outer membrane. BAK then recruits Bcl-2-associated X protein (BAX), which results in macropore formation on the mitochondrial outer membrane [21–23]. Nsp5 is the 3 chymotrypsin-like protease (3CLpro), also called major protease (Mpro). It proteolytically cleaves pp1 and pp1b to release nsp4-11. It is also part of the RTC and has an immunomodulatory effect. IFN- β , ISG56, ISG15 IL-6, and IL-8 expression are suppressed in the presence of nsp5. This is probably because it also cleaves host proteins. Especially those that play an important role in the dsRNA sensing innate immune response cascade. It has been shown that it can cleave the NLRP12 (NLR Family Pyrin Domain Containing 12) and TAB1 (TGF-beta activated kinase 1 (MAP3K7) binding protein 1). It also cleaves amino acids off the N-terminal side of the retinoic acid-inducible gene 1 (RIG-1) protein, thus eliminating its ability to activate the mitochondrial antiviral signalling (MAVS) molecule. Moreover, it also triggers ubiquitination and proteasome-mediated degradation of MAVS. Furthermore, the translocation of phosphorylated IRF3 from the cytosol to the nucleus is also subverted by nsp5, thus blocking the transcription of IFN genes [11,19,24–26]. Nsp6 is also part of the RTC. It inserts into the endoplasmic reticulum (ER) membrane and acts as a connector between the ER and double-membrane vesicles formed by nsp3 and nsp4. Moreover, it filters the flow between the replication organelle and ER. Proteins from the ER are restricted to enter the DMVs. It mediates contact with lipid droplets (LDs) to gain building blocks for DMVs, thus, facilitating viral RNA replication. It also interacts with the sigma receptor, which participates in the stress response of the endoplasmic reticulum. *In vitro*, it has also been shown

that nsp6 suppresses the IFN β , IFN λ 1 and IFN λ 2/3 gene expressions [21,22,24] Nsp7 and nsp8 are important cofactors of nsp12, the RNA-dependent RNA-polymerase (RdRp). Cryo-electron microscopic studies showed that the nsp7-nsp8 complex binds to the finger domain of nsp12 and probably serves as a primase. While another nsp8 sits at the thumb domain, and together with the nsp8 subunit of the previously mentioned nsp7-nsp8 complex, accommodates the exiting RNA. It has also been observed that nsp7 can interact with the Ras-related protein (Rab7A), which is important for vesicle trafficking. In addition, nsp8 can disrupt protein trafficking towards the cell membrane by binding to the signal recognition particle (SRP) [10,27]. Nsp9 has key roles in the replication of viral RNA. It can bind to small RNAs, and in cooperation with nsp13, it can facilitate the generation of the GpppA cap onto the newly synthesised transcripts. Furthermore, it can interact with the eukaryotic translation elongation factor 1A (eEF1A). As the nsp8 and nsp9 can bind to the SRP and suppress membrane protein trafficking upon infection. The host immune response can have a fallback by suppressing SRP-dependent protein secretion. In addition, it has been shown to counteract the nuclear pore complex (NPC) formation by interaction with the NPC's structural component. This interaction causes defective NPC formation and disrupts NF- κ B signalling, which regulates different IFN-stimulated gene expressions [10,27–29]. Nsp10 forms complexes with other non-structural proteins. The nsp10-nsp16 complex methylates at the 2'O-position of the first nucleotide of the 5'-capped RNA genome. This is the last step in the cap-forming cascade. It is crucial for the evasion of the host immune system and protects the RNA from degradation by exoribonucleases. RIG-I can recognise the nascent viral RNA and activates the IFN response. Nsp10 forms a complex with nsp14 as well. Although in this case, nsp10 does not have any influence on the nsp14 methylase activity. It stimulates the exoribonuclease activity of nsp14. The nsp14-nsp10 complex binds to the 3'end of the RNA and helps to maintain replication fidelity. Its RNase activity is enhanced by the nsp12-7-8 complex [7,10,11,30,31]. Nsp12 encodes the RNA-dependent RNA polymerase (RdRp). RdRp is the central enzyme of the RTC; it facilitates the generation of new copies of the viral RNA. Two nsp8 and one nsp7 subunits are essential for nsp12 to form a functional RTC that can effectively incorporate nucleotides *in vitro*. Nsp12 also takes part in the capping of the nascent viral RNA. It has been shown *in vitro* that it also perturbs IFN- β production, so besides its major role, it might influence the host immune response [10,20,30] Nsp13 connects to the RTC. Two copies of nsp13 help the elongation of the replicating viral genome. Besides its helicase activity, it also has RNA 5'-triphosphatase (NTPase) activity. It enables backtracking and proofreading and permits template switching. In addition, IFN inhibition might be mediated by it [10]. The C-terminal

domain of nsp14 has N7-methyltransferase (N7-MTase) activity; thus, it takes part in RNA capping. While its C-terminal end also has a 3'-to-5' exoribonuclease (ExoN) domain, which is responsible for proofreading newly copied viral RNA. Exoribonuclease activity is stimulated by the binding of nsp10. Reports also suggest that it can inhibit IFNs as well [10]. Nsp15 has endoribonuclease activity. It can prevent the RIG-I/MDA5 activation, whose key function is dsRNA (a marker of the viral invasion) sensing and the subsequent triggering of the type-1 interferon expression. It cleaves mostly the 3'-phosphate of uridines, especially when followed by purines. 5'-end of the negative sense genomic RNA ((-) gRNA) contains uridines in a high ratio, but throughout the whole genome, numerous uridines are flanked by purines. Therefore, nsp15 effectively removes the (-) gRNA of the dsRNA, thus eliminating the ligand of RIG-I/MDA5 [10]. Nsp16 is a 2'-O-methyltransferase, which is only active within a complex, formed together with nsp10. It methylates the first nucleotide at the 2'-O-position of a capped RNA strand, the last step of RNA capping[30]

SARS-CoV-2 possesses four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Spike proteins on the viral surface give the well-known crown shape, from which the name, *Coronaviridae* originates. It has two functionally distinct subunits, one is responsible for the viral attachment to the host cell (S1), and the other facilitates the fusion of the viral and host membrane (S2) (Fig. 1). S1 contains the receptor binding domain, which is mainly in a lying-down state, which helps the successful immune evasion. By having a less accessible RBD, SARS-CoV-2 utilises the help of host proteases (TMPRSS2, cathepsins, and furin). The SARS-CoV-2 S1 subunit of spike protein contains a unique cleavage site (RRAR) at the S1/S2 subunit, which can be effectively cleaved by the host furin. Such cleavage site has not been observed in other related CoVs. The high hACE2 affinity and distinct furin cleavage site help the pre-activation and conformational change of the spike, which can lead to successful human host cell attachment (Fig. 1) [3,32]. Upon conformational rearrangements, proteins within the S2 subunit also become functional and mediate host and viral membrane fusion. Following the RBD and ACE2 binding, the fusion peptide is thrust into the host cell membrane. Then three HR1 and three HR2 domains form a six-helix bundle which creates a hairpin that brings the two membranes (viral, host) together (Fig. 1) [11,28]. Besides the spike protein, two other structural proteins are found at the surface of the virus, the E and M proteins (Fig. 2). The E protein participates in the construction of new viruses when they are assembled, and it also forms ion channels. M protein's key role is packing the viral component into the newly assembled viruses. The N protein is found within the virion. It is attached to the viral RNA via

electrostatic interactions (Fig. 2). It modulates the unwinding of the viral genome after entry and can also act against the innate immune system [4,33].

SARS-CoV-2 has several accessory proteins (ORF3a, 3b, 6 7a, 7b, 8, 9a, 9b, and 10). Reports suggest that these proteins can modulate host cell metabolism and antiviral immunity [33]. ORF8 mediates the degradation of major histocompatibility complex (MHC) *in vitro* and prevents the nuclear translocation of IRF3. ORF9b has also been linked to the inhibition of phosphorylation and the nuclear transport of IRF3. ORF7a and ORF3 can also downregulate MHC-I expression. ORF3b, ORF6, and ORF7b are type I interferon antagonists [20,24,33]. Type I and III pathways are also impeded by inhibiting STAT1 and STAT2 phosphorylation and/or nuclear transport. ORF3a, ORF6, ORF7a, and ORF7b have been reported to have such activities [11,24,33]. ORF9b may play a role in inhibiting signalling downstream of RNA sensing [4]. ORF9b also plays a role in cell death, mediated by the inhibition of the anti-apoptotic protein of the Bcl-2 family. In synergy with nsp4, ORF9b facilitated mitochondrial DNS release by forming macropores on the outer mitochondrial membrane and vesicles from the inner membrane [23].

These viral proteins successfully rewire the host processes to produce enormous numbers of viral copies and modify immune responses. Some of these proteins are ideal targets for direct-acting antivirals.

1.1.2. Pathogenesis

Several transmission routes have been reported in the case of SARS-CoV-2. An infected person can infect others directly by respiratory droplets (>5 μm diameter) and/or aerosol particles ($\leq 5 \mu\text{m}$ diameter). Furthermore, besides the airborne infection, faecal-oral route has also been confirmed. Transmission can also happen indirectly via touching infected surfaces. Asymptomatic infections and transmission in the presymptomatic phase added more difficulties to successfully contain the viral spread [34]. Cells that express ACE2 are susceptible to SARS-CoV-2 because the spike protein RBD region has a high affinity for ACE2. This feature results in the attachment of the virus to the host cell. ACE2 is expressed in different cell types in various species, such as human, ferret, rhesus monkey, civet, cat, pangolin, rabbit, pig, and dog. Most of these cells are found in the upper and lower respiratory tracts, airway and alveolar epithelial cells, vascular endothelial cells, alveolar macrophages, and some extrapulmonary tissues, such as the intestinal tracts [3]. Besides the interaction with ACE2, binding to heparan sulfate, integrins, or neuropilin 1 are also reported to play an important role in the attachment

to the host cell [11,33]. To enter the cells, the spike protein of the virus needs to be proteolytically cleaved by host proteases, such as transmembrane protease serine protease 2 (TMPRSS2), cathepsin L, and furin (Fig. 2). Conformational changes are followed by the fusion of the viral and host membranes, which leads to the viral ribonucleoprotein release into the host cytosol. The released RNA is then first translated into two polyproteins, pp1a and pp1ab (Fig.2). These two polyproteins are then processed into several non-structural proteins by nsp3 and nsp5. The released nsps then form the RTC and facilitate immune evasion. Nsps are also responsible for forming different membrane structures (double-membrane vesicle, convoluted membranes, double-membrane spherule) to create a protective microenvironment for viral genome replication and transcription. Possessing one of the largest RNA genomes, its polymerisation is very complex. Coding its own protein with exoribonuclease (nsp14) activity gains high replication fidelity. Increased processivity is acquired by the coordinated work of RdRp and other proteins (nsp7, nsp8). By the RTC, different subgenomic mRNAs are transcribed, which are subsequently translated into structural proteins of the virus (E, M, N, S) (Fig. 2). The newly generated genomic viral RNAs interact with the nucleocapsid protein. Then, together with the spike, envelope, and viral membrane proteins, they form new viruses in the endoplasmic reticulum Golgi intermediate compartment (ERGIC). Ultimately, the newly assembled, matured viruses egress from the host cells through exocytosis (Fig. 2). To date, two ways have been proposed as secretory mechanisms. One is the classical exocytosis pathway and the other is the incorporation into deacidified lysosomes [3,33,34].

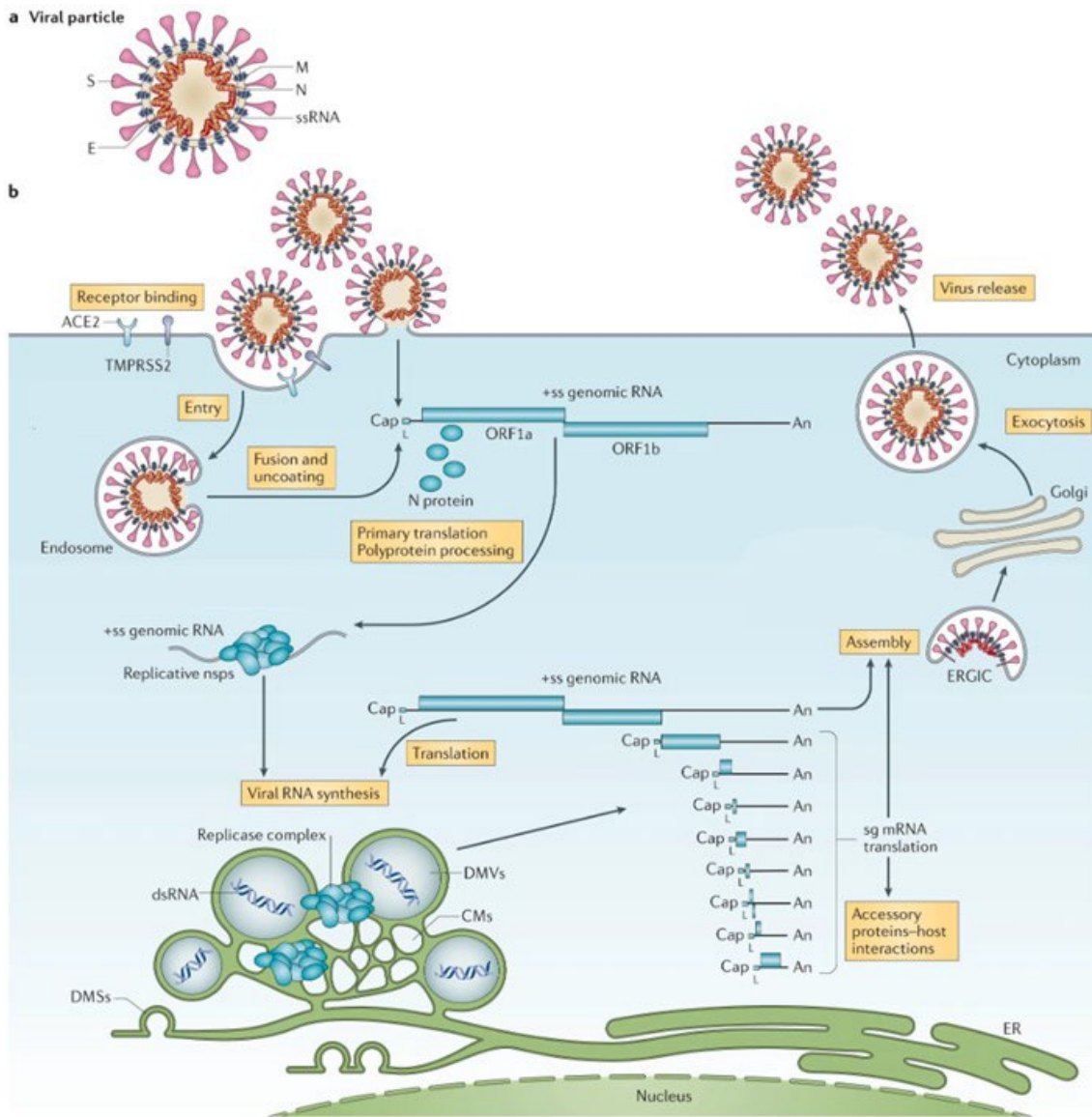


Figure 2. SARS-CoV-2 replication in host cell. Cells expressing ACE2 are susceptible to SARS-CoV-2. Viral S protein can bind to ACE2 with high affinity. After the virus is attached to the cell surface, the viral and cell membranes fuse. As a result, the viral genome enters the cytoplasm, where translation and transcription occur. Viral proteins can manipulate the host and thus produce new viral copies. After the precisely coordinated work by the viral and host proteins, new viruses assemble and mature, then egress from the host cell to infect other cells. Abbreviations: S-spike, M-membrane, N-nucleocapsid, E-envelope, ssRNA-single-stranded RNA, dsRNA-double-stranded RNA, ORF-open reading frame, DMV-double-membrane vesicle, CM-convoluted membranes, DMS-double-membrane spherule, ERGIC-endoplasmic reticulum-Golgi intermediate compartment. Figure source: Copyright © 2020, Springer Nature Limited [11].

Manifestations of SARS-CoV-2 infection can be asymptomatic, mild, moderate, and severe. The disease onset most commonly starts after a 1-14-day-long incubation period. The most common symptoms are sore throat, fatigue, fever, dry cough, headache, and olfactory and

taste disorders. Vomiting and diarrhoea also occur in several cases. Histopathological changes were detected mainly in the lungs of SARS-CoV-2-infected patient. One of the most severe reactions is the cytokine storm syndrome which is considered the leading cause of death from COVID-19. During cytokine storm, various tissues and cells uncontrollably produce several inflammatory cytokines. This drives other immune cells to the sites of inflammation, thereby cascading the exponential growth of the inflammatory response. It can result in acute respiratory distress syndrome and multiple organ failure (by the produced granulysin, perforin, for instance). In severe COVID-19 patients, highly elevated levels of IL-6 have been observed. High levels of IL-6 can enhance vascular permeability which leads to the rapid spread of inflammation and can cause tissue damage. IL-1 β level is also elevated, which causes, for instance, pyroptosis, which has been linked to COVID-19 too. Pyroptosis is an inflammatory form of programmed cell death. IL-6 and IL-1 β trigger complement production, leading to vascular permeability [3,35]. Moreover, the infection can also have long-term effects (long COVID or post-COVID conditions). To date, multiple, newly arisen conditions have been documented to occur in recovered SARS-CoV-2 patients. The long list of adverse outcomes includes cardiovascular, cerebrovascular diseases, myalgic encephalomyelitis/chronic fatigue syndrome, type 2 diabetes, dysautonomia, and cognitive impairment, and thrombotic disorders. These can last weeks, months or even years. Gastrointestinal and respiratory conditions usually wane in a short period. In contrast, neurocognitive symptoms tend to persist for an extended time. Presumably, the causes are diverse and still an area of intense inquiry [34,36].

1.2. Antiviral agents

In the late 1880s, Dimitri Ivanovsky and Martinus W. Beijerinck described a nonbacterial pathogen, a '*Contagium vivum fluidum*' that infected tobacco plants [37]. Since then, numerous viruses have been described, and the capability of human infections has been proved in the case of more than 220 viruses [38]. In 2019, the list of ten global health threats by the World Health Organization contained various viruses. It included the threat of a pandemic caused by an influenza virus. It lists the human immunodeficiency virus (HIV), dengue virus (DENV), ebola virus (EBOV) and other high-threat viruses such as the CoVs, nipah virus (NiV), zika virus (ZIKV) [39]. Effective vaccines have led or might lead to eradicate viral diseases, for instance, smallpox, polio, measles, rubella, and mumps. However, in the case of most viruses, developing an effective vaccine is challenging, and the so-called vaccine hesitancy makes it even harder to keep pace with viral diseases. To date, only two viral infections have been eradicated globally:

smallpox (1980) and rinderpest (2011) [40]. Besides vaccine development (prevention), there is still a considerable need for effective antiviral therapies (e.g., chemotherapeutic agents, antibodies, and medicines from natural sources) to halt severe viral diseases [38].

The first antiviral drug, idoxuridine was approved in 1963. Since then, many new antiviral drugs have been clinically approved (Fig. 3). However, drug development against viruses is relatively slow and expensive because it costs over 2 billion dollars and 8-12 years to develop a therapeutic agent [41].

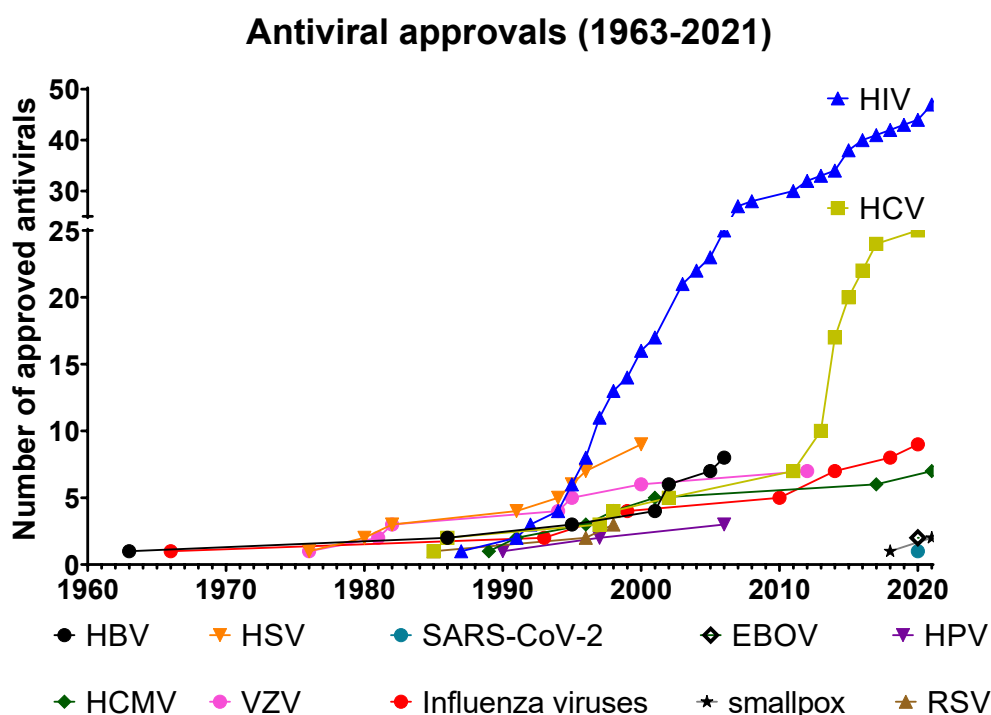


Figure 3. Timeline of antiviral approvals. Since 1963, many antiviral drugs have been approved. During the short history of antiviral development, there were some notable milestones. In the late 1970s, acyclic nucleoside analogues (e.g., acyclovir) that could interfere with viral DNA synthesis with enormous tolerance were discovered. In the 1980s, a new devastating viral disease, AIDS appeared. This encouraged an intensive search for anti-HIV compounds. Anti-HIV medications still lead the list of antiviral agents. Recent technical innovations such as genome sequencing and structure-based drug design have also increased the effectiveness of antiviral R&D. The current COVID-19 pandemic has boosted the research on chemotherapeutic antivirals. Interestingly, two antivirals have recently been approved against an eradicated virus, smallpox [42–45].

In principle, antivirals target either a viral protein (direct-acting antivirals) or a cellular protein (host-directed antivirals). Both approaches have their benefits and drawbacks. For example, drugs that target cellular protein(s) can have undesirable side effects but are less prone to antiviral drug resistance development. On the contrary, direct-acting antivirals can be well

tolerated, but the likelihood of resistance development is higher. The preferred method depends on the nature of the virus or its host cell properties. The currently available antiviral drugs can be divided into several functional groups (Fig. 4). Drugs can inhibit one of the steps of viral replication cycle or even target several targets (mono-and combination therapies). It includes virus attachment, entry, viral DNA or RNA synthesis, and viral egress. Antivirals can block viral proteins such as polymerases, proteases, neuraminidase, and integrases. Entry inhibitors (enfuvirtide, maraviroc etc.) target non-enzymatic processes in the viral replication cycle. They are primarily peptides or antibodies that are antagonists of the host cell receptors. Polymerase inhibitors can be divided into (a) nucleoside analogues and (b) non-nucleoside allosteric inhibitors. Allosteric inhibitors (nevirapine, doravirine etc.) bind to the polymerase and cause conformational changes that impair the enzyme function. Nucleoside analogues (azidothymidine/ zidovudine, didanosine, acyclovir, ganciclovir, remdesivir, ribavirin etc.) are chemically synthesized derivatives of purine and pyrimidine bases. Chemical alterations can be in the base or sugar component of the compound. Altogether, different types of nucleoside analogues are the most common antivirals. These derivatives abrogate the replication of the viral genome. They can act as chain terminators or they can be incorporated into the growing genome copy of the virus, causing detrimental mutations. Protease inhibitors (saquinavir, ritonavir, lopinavir, darunavir etc.) block the maturation of viral polyproteins. Neuraminidase inhibitors (oseltamivir, zanamivir etc.) competitively target the active site of influenza virus neuraminidase, thus blocking the enzymatic cleavage of the terminal sialic acid residues. Due to this interaction, influenza virus particles remain bound to the host cell membrane. Integrase inhibitors (raltegravir, dolutegravir, cabotegravir, etc) block the integration of viral genome into the human chromosomal DNA. To date, these are only available commercially against HIV. Host cellular enzymes such as inosine 5'-monophosphate (IMP) dehydrogenase and S-adenosylhomocysteine (SAH) hydrolase, are also preferred targets. IMP is inhibited for instance by ribavirin and mycophenolic acid. Potent inhibitors of SAH are the carbocyclic 3-deazaadenosine, and 3-deazaneplanoci A [46]. Other host targets, for example, cyclophilins, glucosidase, different heat shock proteins, eukaryotic initiation factor 2 α and various kinases, were in the scope of antiviral drug designs. One of the best examples of host-directed antivirals is the interferons (e.g., interferon α -2 B, interferon α -N3 etc.)

Functional groups of approved antiviral drugs

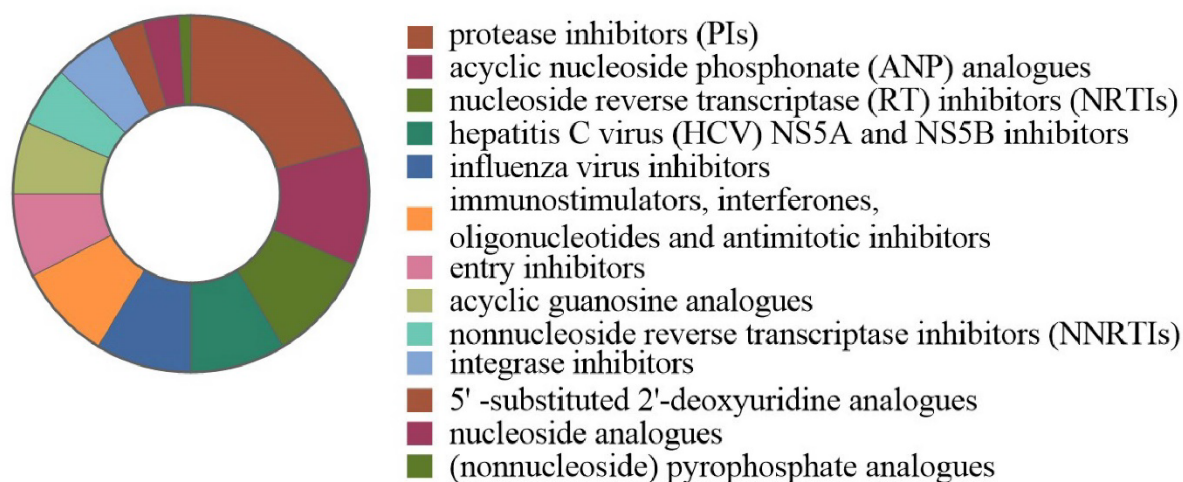


Figure 4. Functional groups of approved antiviral drugs. There are several types of commercially available therapeutic agents to combat viral diseases. Most of them are virus-targeting agents. [42,44].

One of the major challenges in antiviral therapy is the newly evolved resistance. Prolonged antiviral therapy can lead to the accumulation of different mutations, *e.g.*, in the viral polymerase or reverse transcriptase, and eventually new drug-resistant virus variants evolve. The error-prone nature of viral polymerases can also ease the quick adaptation. To overcome this problem, a good approach can be if the compounds, preferably with different mode of action, can be combined to fight the viral infection. Furthermore, constant monitoring and more studies should be conducted to examine the evolving resistant viruses. [47,48].

Additionally, from time to time, viruses re-emerge or unknown viruses emerge, such as DENV, EBOV, SARS-CoV-1, SARS-CoV-2, and MERS-CoV. Currently, most of the approved antiviral drugs are direct-acting agents that target viral enzymes (proteases, polymerases). These agents have a narrow spectrum of coverage; thus, they could be more effective against emerging and re-emerging viruses. Broad-spectrum antivirals can be the key to combat against these viruses. By developing such agents, the cost and time of drug development can be reduced; administering a broad-spectrum antiviral before the viral threat has been precisely diagnosed can help reduce the fatality. One of the first broad-spectrum antivirals is ribavirin, which inhibits viral RNA and DNA synthesis. In the preclinical phases, ribavirin proved to be effective against different viruses (*e.g.*, lassa virus (LASV), DENV, sindbis virus (SINV), poliovirus (PV), and vaccinia virus (VACV)). However, clinically, only hepatitis C (HCV) and respiratory syncytial virus (RSV) are treated with it [41,49,50]. Besides

ribavirin, other nucleoside analogues proved to have broad-spectrum antiviral activity and can be used clinically, such as galidesivir (BCX4430), favipiravir (T-705), and brincidofovir (CMX001). The rational drug design of broad-spectrum antivirals is aggravated due to the broad structural and sequential diversity of viral proteins. Therefore, another approach is to target host proteins or pathways required for viral replication of different viruses. Cyclophilin A inhibitors showed promising preclinical results, for instance, against HIV, DENV, CoVs, and influenza A virus (IAV). Alisporivir (DEB025) and SCY-635 have already been involved in phase II/III clinical trials and had encouraging results for HCV treatment [41,51,52].

Furthermore, another good way to obtain effective antivirals is to examine drugs that target host functions that are also required for viral replication (drug repurposing). Several anticancer drugs that inhibit different kinases, such as cyclin G-associated kinase (GAK) and AP2-associated protein kinase 1 (AAK1), proved to have broad antiviral activity. Erlotinib, sunitinib, dasatinib, imatinib, and nilotinib proved to be effective *in vivo* and *in vitro* against, for example, EBOV, DENV, HIV, and HCV [41,53,54]. Nitazoxanide, used for treating parasitic diarrhoea, also had antiviral effects in preclinical studies. In clinical studies, it shortened the duration of flu symptoms. Also, lipid metabolism can be targeted, for example utilising statins to block viral replication. Furthermore, inhibiting endosomal acidification using, e.g., chloroquine (CQ) or hydroxychloroquine sulfate (HCQ), which are antimalarial and antirheumatic-drugs, also proved beneficial against certain viral infections [41]. Repurposing drugs, which are used for the treatment of chronic disease, can have a less toxic effect in a shorter antiviral therapy. Also, resistance development is less frequent and the resistance level is lower in the case of host-targeted medicines. Unfortunately, the *in vitro* antiviral effects cannot be reproduced either *in vivo* or in clinical settings. The cellular network of interactions is very complex; unravelling the exact mode of action of a drug is crucial and might help to overcome the ineffective translation into clinical settings [41,55].

Taken together, to be able to fight more effectively against emerging viral diseases, more studies are needed, including the generation of more lead compounds, understanding the complex cellular pathways and mode of action, and testing the antiviral activity of already registered and approved drugs.

1.2.1. Antiviral drugs against SARS-CoV-2

Different approaches have been applied to find effective therapeutics against COVID-19. Direct-acting and host-directed antivirals are also being tested in preclinical and clinical

settings. COVID-19 therapies target the SARS-CoV-2 replication cycle and the other symptoms triggered by the infection (e.g., cytokine storm, long-COVID). Umifenovir[®] (arbidol), camostat mesylate, CQ, and HCQ are just a few examples that have been proposed to block viral entry. Viral entry inhibitors also compose monoclonal antibodies, such as Regkirona[®] (regdanvimab), Xevudy[®] (sotrovimab), Evusheld[®] (tixagevimab, cilgavimab), and Ronapreve[®] (casirivimab, imdevimab) (Fig. 5 A, B, and C). Viral replication inhibitors target RdRp (remdesivir, favipiravir, molnupiravir etc.) or other viral proteins, like 3CLpro (e.g., lopinavir and ritonavir) (Fig. 5 D and E). Several immunomodulatory agents aimed to eliminate the SARS-CoV-2-induced cytokine storm. Dexamethasone, tocilizumab, sarilumab, bevacizumab, eculizumab, and interferons have all been tested to relieve the inflammatory response in COVID-19 patients (Fig. 5 G). Convalescent plasma treatment has also been investigated.

Amongst the numerous ways to seek effective antivirals, a huge number of small molecules have been tested to find potent direct-acting antivirals. RNA-dependent RNA polymerisation is a very tempting target for antivirals. Different nucleoside analogues tend to have a broad-spectrum inhibitory activity on RdRps (Fig. 5 E) [28]. Remdesivir (GS-5734) was the first antiviral that earned emergency use authorisation (EUA) to treat severe COVID-19. It was originally developed against the HCV infection by Gilead Sciences. It is a modified adenosine nucleoside analogue that can cause chain termination. It can avoid the SARS-CoV-2 nsp14 exoribonuclease activity since following the remdesivir, additional nucleoside triphosphates are built in before the chain termination [6,28]. Direct-acting antivirals should be added at the early phase of the infection, as viral replication peaks a bit after the onset of the symptoms. Remdesivir is administered intravenously, which can complicate the treatment. Consequently, oral antivirals are important for effective COVID-19 treatment in an outpatient setting, as the therapy can start early. Favipiravir (T-705) was proposed to be a good candidate to halt the severity of COVID-19. It is a purine analogue that can cause lethal mutagenesis. Its antiviral potential was first identified during an antiviral screening against influenza viruses conducted by Toyama Chemical. Since the beginning of the pandemic, several trials have evaluated the effectiveness of this drug against SARS-CoV-2. The results are somewhat controversial, a clear conclusion cannot be drawn from the available results [6,28,56]. Sofosbuvir and bennifosbuvir (termed AT-527) are currently used for treating HCV infection. Both were predicted to be beneficial against SARS-CoV-2, although, to date, sufficient data are not available to strongly support the usage of those drugs in COVID-19 patients [6,28]. Recently, amongst the RdRp inhibitors, molnupiravir has shown promising results. It was developed against influenza

viruses. The inhibitory activity is presumably based on the generation of many mutations, just like in the case of favipiravir. The promiscuous base pairing cannot be overruled by nsp14, and eventually, an error catastrophe occurs. It is a well-tolerated drug; outpatients can also be treated with it [6,28]. However, concerns have been raised about the evolution of drug resistance variants under the selective pressure of the drug. Fortunately, a recent study proves it would be unlikely [57]. Besides the RdRp, other viral protein activities have also been targeted (Fig. 5 D). Currently, one of the most promising drugs that can be administered orally is a 3CLpro inhibitor, Paxlovid™ (nirmatrelvir, ritonavir). It can covalently bind to the main protease and outcompete its natural substrates. Studies with nirmatrelvir started during the previous SARS-CoV epidemic. During the SARS-CoV-2 pandemic, Pfizer continued to study these compounds, both in oral and intravenous formulations. It became the first oral antiviral to get EUA. Ritonavir was added to nirmatrelvir (Paxlovid™) to enhance the pharmacokinetic profile and half-life of the drug [6,58]. Several drugs could inhibit one of the steps of viral entry. Monoclonal antibodies can cover the spike protein, especially regions of the S1 subunit. Thus, it cannot interact with the ACE2 receptor (Fig. 5 A). Serine protease inhibitors, such as camostat, nafamostat, aprotinin, and bromhexine, can inhibit TMPRSS2, and subsequently, conformational changes in spike protein cannot occur (Fig. 5 B). However, these substances showed poor clinical applicability against COVID-19. SARS-CoV-2 can enter the host cell via endocytic pathways too. Endosomal trafficking can be blocked *in vitro*, for instance, by CQ, HCQ, and teicoplanin (Fig. 5 C). The effectiveness of CQ and HCQ were investigated in numerous clinical trials. Several trials disproved the efficacy of these CQ and HCQ. Therefore, the use of these drugs in clinical settings was discontinued in many countries [28].

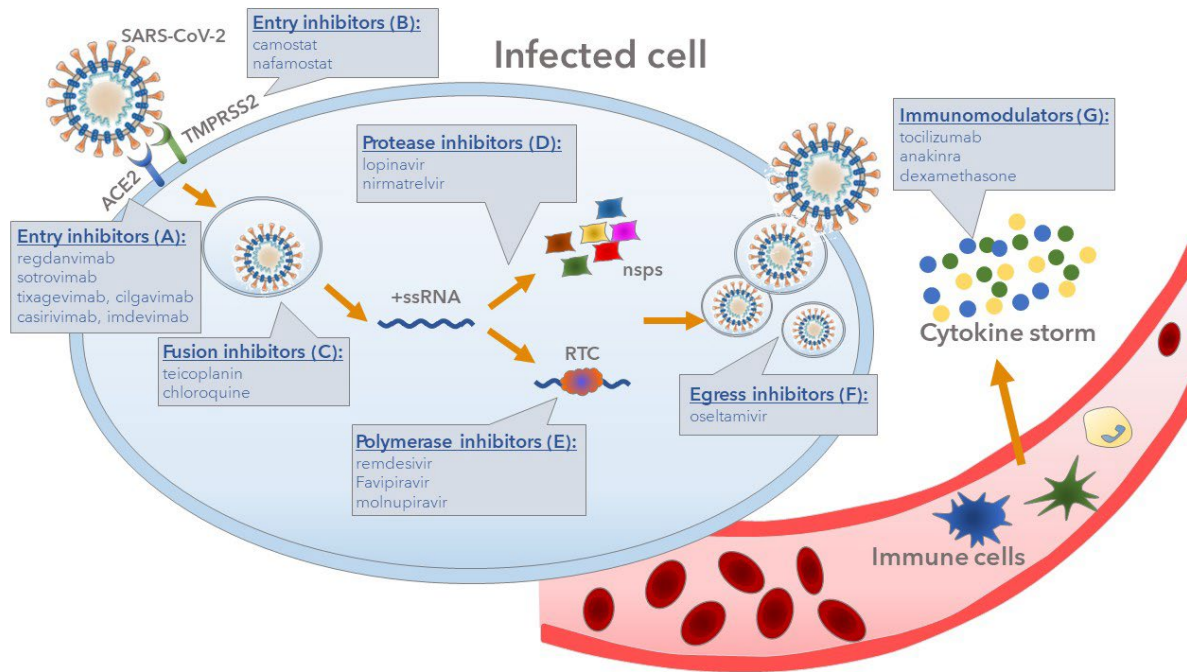


Figure 5. Targets of authorised COVID-19 therapeutics and a few proposed antivirals. Since the declaration of the pandemic, numerous clinical trials have started to confirm the results of preclinical studies. Several studies were conducted to elicit the exact mechanism of the *I* replication. Thus, more insights were gained for rational drug designs. Throughout the *nt* years (2020-2023.) a lot of drugs were found to effectively inhibit directly the virus or host proprocesses significant to the viral pathogenesis. Unfortunately, some purported potent inhibitors proved to be ineffective in clinical settings. The current FDA/EMA authorised drugs target the entry, viral proteins and cytokine sto [59,60].

To date, several drugs have been authorised to treat COVID-19. The European Medicines Agency (EMA) authorised for use the Evusheld[®] (tixagevimab, cilgavimab), the Regkirona[®] (regdanvimab), the REGEN-COV[®] (casirivimab, imdevimab), the Xevudy[®] (sotrovimab), the Kineret[®] (anakinra), the RoActemra[®] (tocilizumab), the Veklury[®] (remdesivir) and the Paxlovid[™] (nirmatrelvir, ritonavir) [61]. United States Food and Drug Administration (FDA) approved three drugs, Actemra[®] (tocilizumab), Veklury[®] (remdesivir) and Olumiant[®] (baricitinib). Furthermore, FDA authorised for emergency use several other products for the treatment of COVID-19; the bebtelovimab, the Evusheld[®] (tixagevimab, cilgavimab), REGEN-COV[®] (casirivimab, imdevimab), the Xevudy[®] (sotrovimab), the bamlanivimab, the etesevimab, the Paxlovid[™] (nirmatrelvir, ritonavir), the Lagevrio[®] (molnupiravir), and Kineret[®] (anakinra) [62] Except anakinra and tocilizumab, all target the viral replication (Fig. 5).

Nirmatrelvir inhibits the Mpro protease. It is administered in combination with ritonavir which serves as a pharmacokinetic booster. Remdesivir and molnupiravir have detrimental effects on viral replication. Remdesivir binds to the RdRp which results in the premature termination of RNA transcription. While molnupiravir is incorporated into the growing copies of viral RNA that causes lethal mutagenesis. The remaining drugs of the lists above are monoclonal antibodies that block the interaction between the viral spike protein and ACE2, thus impeding the viral attachment to the host membrane. Currently, there are still numerous ongoing trials to evaluate the safety and effectiveness of a vast variety of therapeutics in different patient groups. Immunocompromised people who cannot respond appropriately to vaccination, vaccine hesitancy, and newly emerging variants lead to vaccination failure. Long-COVID can also pose a threat to infected patients. Therefore, developing effective therapies is still utterly important to properly manage COVID-19.

2. Objectives

The main aim of the work was to find compounds that effectively inhibit the newly emerged coronavirus, the SARS-CoV-2. The pace of the transmission and the damage caused by the disease on the economics and health care system was daunting. It caused an unprecedented collaboration between different sectors. We also aimed to gain knowledge and enlarge the list of pharmacological agents and newly synthesised compounds with potent antiviral activity against SARS-CoV-2. Our research group had access to well-equipped laboratories, and even work in a high containment laboratory (BSL-4) was available; thus, safe work was guaranteed with the infectious virus. To be able to start the experiments, first, we needed to:

1. Successfully propagate the SARS-CoV-2 to generate viral stocks.
2. Determine the titer of the generated viral stocks.
3. Gain more experience working in a protective suit in a BSL-4 laboratory.

Our research group did not have an optimised system to determine the antiviral effectivity of different compounds. Thus, we needed to:

4. Establish a reliable antiviral testing system with our currently available resources.
5. Build and maintain good collaboration with researchers from different disciplines.

An enormous amount of work was done worldwide to characterise the pathomechanism of COVID-19 and to find ways to effectively treat the infected patient with mild, moderate, and severe symptoms. Moreover, strategies to prevent infection were also published day by day. To be able to provide reliable information at high quality as quickly as possible, we:

6. Focused on testing compounds that were predicted to be effective, based on either *in silico* analyses or literature data.
7. Prioritised drugs that already had well-described pharmacokinetic profiles to mobilise them for treating COVID-19.

Finally, proper lead molecules are essential starting points for drug development campaigns. To advance in finding new compounds to be developed into good clinical candidates, we aimed to:

8. Validate the reliability of a novel computational approach that can ease the search for functional building blocks.

9. Test different newly synthesised derivatives of glycopeptide antibiotics to find the most suitable modifications for antiviral activity.

3. Materials and methods

3.1. Cell lines

Vero E6 (ECACC, UK), derived from African Green monkey kidney epithelial cells, is a widely used cell model in virological research. SARS-CoV-2 can effectively infect and replicate in Vero E6 [63,64]. Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, L-Glutamine, and sodium pyruvate (Lonza Group Ltd, Switzerland) supplemented with 10 % volume per volume (v/v) heat-inactivated fetal bovine serum (HI FBS) (Gibco, Thermo Fisher Scientific Inc., USA) and 1 % (v/v) penicillin-streptomycin (PS) (Lonza Group Ltd, Switzerland) was used to maintain Vero E6. A549 Dual™ hACE2-TMPRSS2 cells (InvivoGen, USA) which overexpress human ACE2 receptor and TMPRSS2, were maintained in DMEM (Lonza, Switzerland) supplemented with 10 % (v/v) HI FBS (Gibco, Thermo Fisher Scientific Inc., USA), 1 % (v/v) PS (Lonza Group Ltd, Switzerland), 100 µg/ml hygromycin (InvivoGen, USA) and 0.5 µg/ml puromycin (InvivoGen, USA). Both cell lines were maintained in a humidified cell culture incubator at 37 °C, supplemented with 5 % CO₂. Before the experiments, the cells were dissociated from the flasks using a solution containing trypsin and versene (Lonza Group Ltd, Switzerland). After incubation with the dissociation solution, FBS containing cell culture media was added. Then the cells were centrifuged at 200 g for 5 minutes. To count the cells, Brand™ Bürker counting chamber (Brand, Germany) was used, and the cells were seeded in a density appropriate to the cell culture plate utilised in the experiment. Mycoplasma contamination was routinely checked with PCR [65].

3.2. Virus

A Hungarian SARS-CoV-2 isolate was used in our experiments. B.1.5 (G) isolate (GISAID accession number: EPI_ISL_483637) contains the D614G mutation in its spike [66]. The virus was propagated in Vero E6 cells. Vero E6 cells were seeded in a T75 cell culture flask, one day before the experiment. 80 %-90 % confluent cells were infected with 1 ml of the virus suspension for an hour. After one hour, the cell culture media was supplemented with 14 ml DMEM (Lonza Group Ltd, Switzerland), containing 2 % (v/v) HI FBS (Gibco, Thermo Fisher Scientific Inc., USA), 1% (v/v) PS (Lonza Group Ltd, Switzerland). Infected cells were incubated for two days in a humidified cell culture incubator. Forty-eight hours post-infection, cells were inspected under an inverted light microscope (Eclipse Ti2-U, Nikon, Japan). When visible virus replication was detected, judged by the formed syncytiums and the increased number of death cells, the supernatant was collected. The whole supernatant was aliquoted into

screw capped microcentrifuge tubes. Aliquots were stored at -80 °C. Nanopore sequencing was used to identify the genomic characterisation of the virus used.

3.2.1. Infective viral titer measurement

Infective viral titer was expressed in 50 % tissue culture infectious dose (TCID₅₀) value. Briefly, a ten-fold serial dilution was prepared from the virus stock. In a 96-well plate, Vero E6 cells were infected. Each column contained 8 replicates from one dilution step (10⁻¹-10⁻¹⁰). Cells in the first column were infected with undiluted virus suspension. At the same time, cells in the last column were not infected. The cells were infected for one hour, then the virus suspension was replaced by DMEM (Lonza Group Ltd, Switzerland) supplemented with 1 % PS (Lonza Group Ltd, Switzerland) and 2 % HI FBS (Gibco, Thermo Fisher Scientific Inc., USA). After seventy-two hours, visible signs of infection (syncytium, apoptotic/necrotic cells) were checked by inspecting the cells under a light microscope (Eclipse Ti2-U, Nikon, Japan). Positive and negative wells were counted, and TCID₅₀ was determined using a TCID₅₀ calculator spreadsheet, which utilises the Spearman-Kärber algorithm [67].

3.3. Compounds

Remdesivir was purchased from Excenen PharmaTech (China) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). HCQ (TCI Europe Chemical Research, Belgium) was diluted in nuclease-free water (New England Biolabs Inc., USA) and filtered through a 0.22 µm sterile syringe filter. Telmisartan, azelastine hydrochloride (HCl), axitinib, amiloride HCl, maraviroc, tolbutamide, clevidipine butyrate, metoprolol, esmolol, losartan, ziprasidone HCl were obtained from Selleckchem (USA) in 10 mM DMSO solution. All solutions were stored according to the manufacturer's recommendation. Rucaparib was a gift from Dr Thomas Harding (Clovis Oncology, USA). Olaparib, talazoparib, and methylene blue were purchased from Sigma-Aldrich (USA) and dissolved in DMSO (Sigma-Aldrich, USA). All of the newly synthesised glycopeptide antibiotic derivatives from the University of Debrecen were also dissolved in DMSO. Ten mM stock solutions were stored at -20 °C. All dissolved compounds were stored in sterile Eppendorf tubes secured with parafilm to avoid oxidation. Fragments, detected by the SpotXplorer were obtained from BioBlocks (USA) and other vendors and dissolved in DMSO (Sigma-Aldrich, USA).

3.4. Cytotoxicity

CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega Corp., USA) was used to assess the cytotoxic effect of the drugs at the described concentrations. The assay is based on the premise that the Ultra-Glow[™] rLuciferase interacts with the ATP, which is produced by metabolically active cells. As a result, oxyluciferin and a luminescent signal are produced that can be detected using a luminometer. Cell viability was measured according to the manufacturer's recommended instructions. Briefly, the treated cells were incubated for 48 hours at 37 °C in a cell culture CO₂ incubator. CellTiter-Glo[®] reagent was added to the wells. Following ten minutes, the luminescent signal was measured using a PerkinElmer 2300 EnSpire Multimode Plate Reader (PerkinElmer Inc., USA). In case of >90 % cell viability (compared to the control, untreated cell viability), the compound at the examined concentration was considered non-toxic.

3.5. *In vitro* infection

One day before the infection, cells were seeded in a cell culture plate. When the cells reached 80 % confluency, the cell culture plates were transferred to the BSL-4 laboratory at the Szentágothai Research Centre, University of Pécs. Cells were treated with different compounds at the desired, non-toxic concentrations. Immediately after adding the compound-containing media to the cells, the cells were infected with a Hungarian SARS-CoV-2 isolate (MOI: 0.01) (co-administration). After 30 min incubation at 37 °C in a 5 % CO₂ atmosphere, the virus-containing media was replaced with fresh media containing the compounds at appropriate concentrations, DMEM (Lonza Group Ltd, Switzerland), 1 % PS (Lonza Group Ltd, Switzerland) and 2 % HI FBS (Gibco, Thermo Fisher Scientific Inc., USA). Azelastine HCl was tested in a therapeutic setting as well. In that case, the Vero E6 cells were infected, and 30 minutes later, the virus suspension was discarded. Then the cells were incubated with cell culture media supplemented with azelastine HCl at the desired concentrations. Forty-eight hours post-infection virus-induced cytopathic effect was examined under an inverted microscope (Eclipse Ti2-U, Nikon, Japan). Supernatants for further analysis were stored at -80 °C.

3.6. Determination of antiviral efficacy

3.6.1. CPE reduction

SARS-CoV-2-induced cell death was inspected under an inverted microscope (Eclipse Ti2-U, Nikon, Japan) to check whether the infection was successful and to estimate the effectiveness of the treatment. Cytopathogenic effect (CPE) was scored; ++++ represented the CPE equal to the positive control, meaning 100 %. +++ was when the infection was 75 %, ++ was when around 50 % infection was detectable compared to the positive control, + was when only a few spots of the monolayer showed CPE and 'No CPE' was noted down, in case of undetectable CPE.

3.6.2. Nucleic acid extraction

Total RNA was extracted from the whole supernatant (100 μ L) using Monarch[®] Total RNA Miniprep Kit (New England Biolabs Inc., USA) or by using an EXM3000 nucleic acid isolation system (Zybio, China) in the BSL-4 facility of the Szentágotthai Research Centre, University of Pécs. Monarch[®] Total RNA Miniprep Kit is a silica-column-based extraction method. Briefly, each sample (100 μ L) was mixed with lysis buffer (300 μ L), and then genomic DNA (gDNA) was bound on a specific gDNA removal column, which was then removed. 96 % ethanol was added to the flow-through. Then, RNA from the flow-through was bound to the RNA purification column. After optimising the binding and further purifying the sample, RNA was eluted from the column with nuclease-free water (50 μ M). EXM3000 nucleic acid isolation system is based on the magnetic bead extraction method. In brief, samples were put in the cartridge included in the viral RNA extraction kit (Zybio, China). To each well, 15 μ L Proteinase K was added as well. Then the cartridges were transferred into the EXM3000 extraction robot (Zybio, China). The extraction robot made the lysis, bound the RNA to the magnetic beads, and washed and eluted the RNA in nuclease-free water. RNA extracts (50 μ l) were stored at -80 °C until further analysis.

3.6.3. Droplet-digital PCR (ddPCR)

QX200 Droplet-Digital PCR system (Bio-Rad Laboratories, USA) was utilised to determine the viral copy number [68]. The benefit of this system is that absolute quantification of RNA is possible without the need for a standard curve. Moreover, it is more sensitive than other PCR technologies. A 1-step RT-ddPCR Advanced Kit for Probes by Bio-Rad (USA) was used to prepare the PCR mix. The PCR master mix consisted of 1X supermix, 20 U/ μ l reverse

transcriptase, 15 mM dithiothreitol, 11.1 μ L nuclease-free water, 900 nM forward and reverse primer, 250 nM TaqMan probe and 2 μ L 100 fold diluted (diluent was nuclease-free water) RNA extract. Primers and probe were the SARS-CoV-2 Charité/Berlin RdRp specific oligonucleotide set (Integrated DNA Technologies, USA). The sequence of the forward primer was GTGARATGGTCATGTGTGGCGG, the reverse primer was: CARATGTTAAASACAC-TATTAGCATA and the probe was: FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ. After generating droplets by the QX200 Droplet Generator (Bio-Rad, USA), the reaction mix was transferred into a C1000 Touch Thermal Cycler, and the samples were amplified. One cycle of reverse transcription was made at 50 °C for 60 minutes. The amplification of the reverse transcribed sample was started with a 10-minute-long enzyme activation at 95 °C, then 40 cycles of 30 seconds of denaturation at 95 °C and 1-minute-long annealing and extension at 58 °C followed it. Lastly, the polymerase enzyme was deactivated by a 10-minute-long incubation at 98 °C. After the thermal cycling, the amplicons were stored at 4 °C until the plate was transferred to the QX200 Droplet Reader. QX200 Droplet Reader counted the PCR-positive and PCR-negative droplets. The absolute copy/ μ L value of each sample was calculated automatically by QuantaSoft™ Analysis Pro version 1.0. (Bio-Rad Laboratories, USA) software.

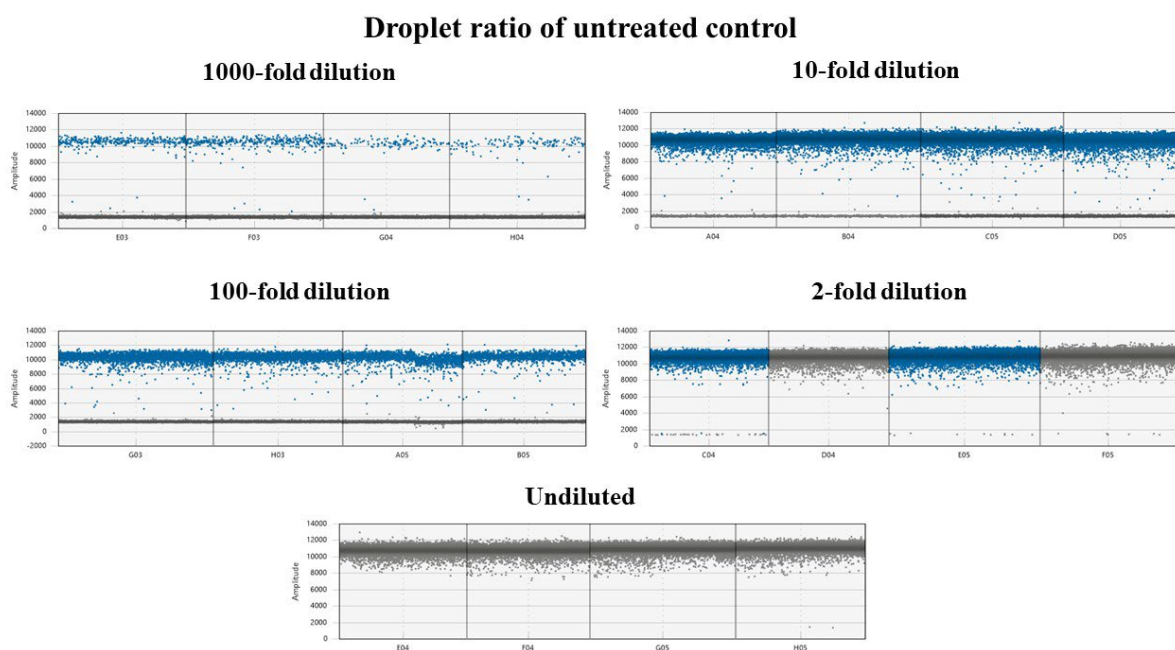
3.6.4. IC₅₀ determination

GraphPad Prism 8 software (GraphPad Software, USA) was used to determine the concentration of a drug needed to inhibit viral replication by 50 % (IC₅₀). The used concentration values were log₁₀ transformed, and then the log(inhibitor) vs response-variable slope (four-parameters) curve fitting (non-linear regression) model was applied to calculate the IC₅₀ values. The IC₅₀ was calculated from at least three replicates of independent experiments [69].

4. Results

Our research group had just started antiviral testing projects when SARS-CoV-2 emerged (at that time, it was termed nCoV19) [70,71]. Our laboratory was not equipped with multimode plate readers; thus, assays based on cell viability or immunostaining were not feasible. Determining the viral titer of differently treated samples using TCID₅₀ or plaque assay would have been insufficient since we needed to test numerous compounds at various concentrations. Fortunately, we had access to a top-notch PCR system (droplet-digital PCR). Since it is highly accurate and provides an absolute count of the amplified sample without the need for standard curves, reverse transcription and amplification of the RNA sample were possible to be done in

one reaction instead of two separate ones; we found it suitable for our antiviral screenings. First, we needed to ensure that the PCR settings were appropriate. Our main concern was the dilution rate of the sample, as in the case of a highly concentrated sample, the positive droplets could interfere with the accuracy of detection. To examine whether we need to dilute the RNA template for the PCR reaction, we compared the detection of 1000-, 100-, 10-, and 2-fold diluted and undiluted RNA extracts of untreated control and treated samples (Fig. 6). Vero E6 cells were treated with a compound that showed reduced viral replication, judged by the lower rate of visible cytopathic effect. The reasoning behind the addition of treated samples was to avoid generating false negative results by the dilution.



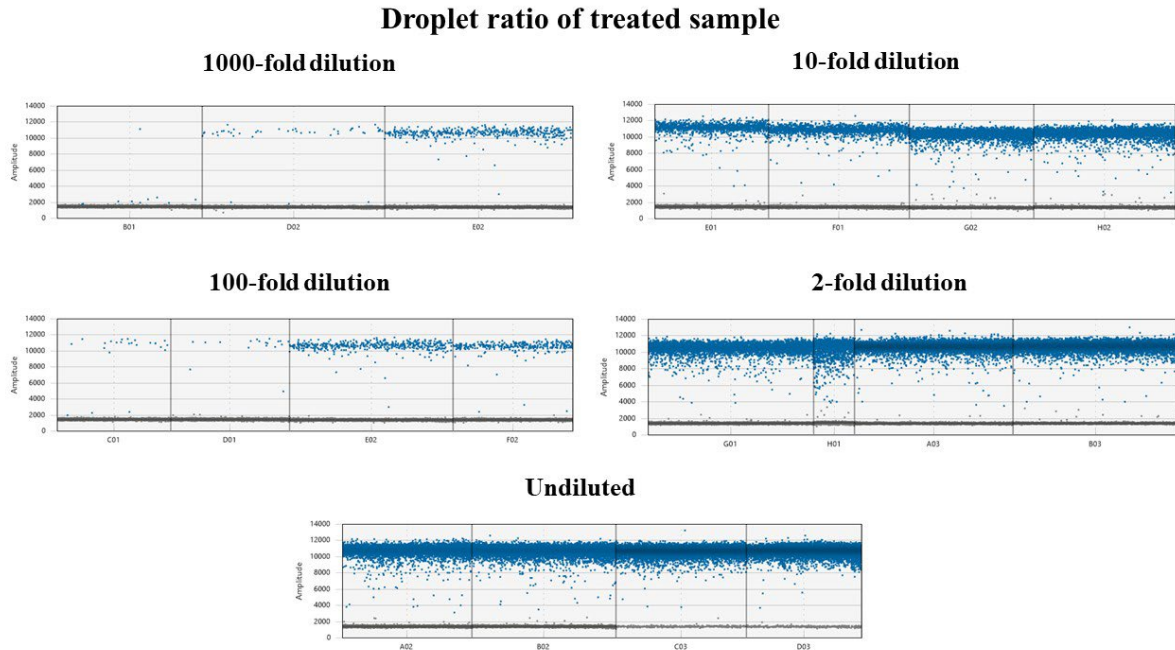


Figure 6. *Fluorescent readouts from a ddPCR analysis of differently diluted templates. We examined the ddPCR detection accuracy of 1000-, 100-, 10-, 2-fold and undiluted samples. The positive and negative droplets were visualised by Quantasoft™ Analysis Pro software. Fluorescent signals were detected by the FAM channel by the QX200 Droplet reader, and the amplitude values were graphed by the software. Droplets emitting signals were represented in blue, and the negative droplets were shown in grey.*

1-D fluorescence amplitude plots were generated by the Quantasoft™ Analysis Pro software. We found that false negative results could be caused by diluting the samples 1000 times. Copy numbers of undiluted, untreated templates could not be determined by the software, even when we set the threshold manually. We experienced the same in the case of some of the 2-fold diluted, untreated samples. 100-, and 10-fold dilution of the samples proved to be safe to use. We decided to dilute the samples 100 times, thereby in case of a need for technical replicates, we would have enough samples left.

When we could generate our viral stocks and set the ddPCR measurement, several studies were published about effective drugs against SARS-CoV-2. To test our method, we selected two compounds that have already been proven to be effective against SARS-CoV-2, HCQ and remdesivir. As remdesivir was dissolved in DMSO, we first needed to confirm that the solvent had no antiviral effect at the concentrations used at the antiviral screens. SARS-CoV-2 could effectively replicate in Vero E6 cells in the presence of DMSO. Remdesivir and HCQ protected Vero E6 cells at specific concentrations (Fig. 7 and 8).

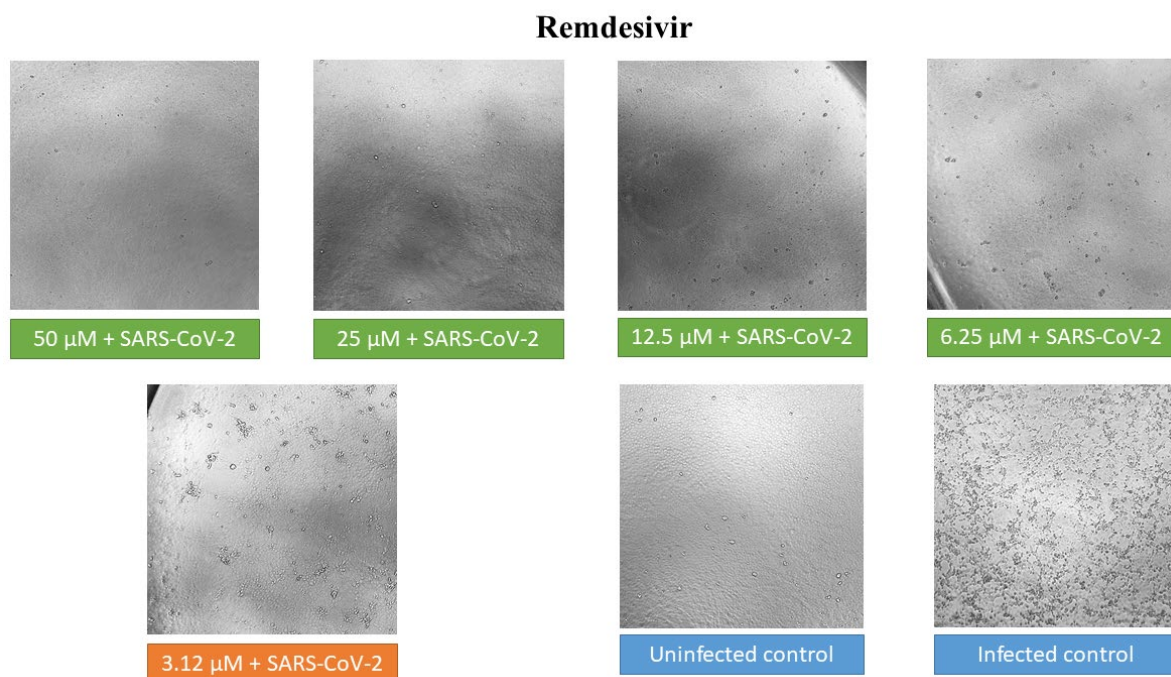


Figure 7. Microscopic evaluation of remdesivir antiviral effect. Vero E6 cells were treated and infected for 30 minutes, and then the remdesivir-supplemented, virus-containing media was replaced with only remdesivir-containing cell culture media. Cells were inspected under a light microscope (10X magnification). Rounded and dense cells display virus-induced cell damage; when it is progressed, the monolayer is also ripped.

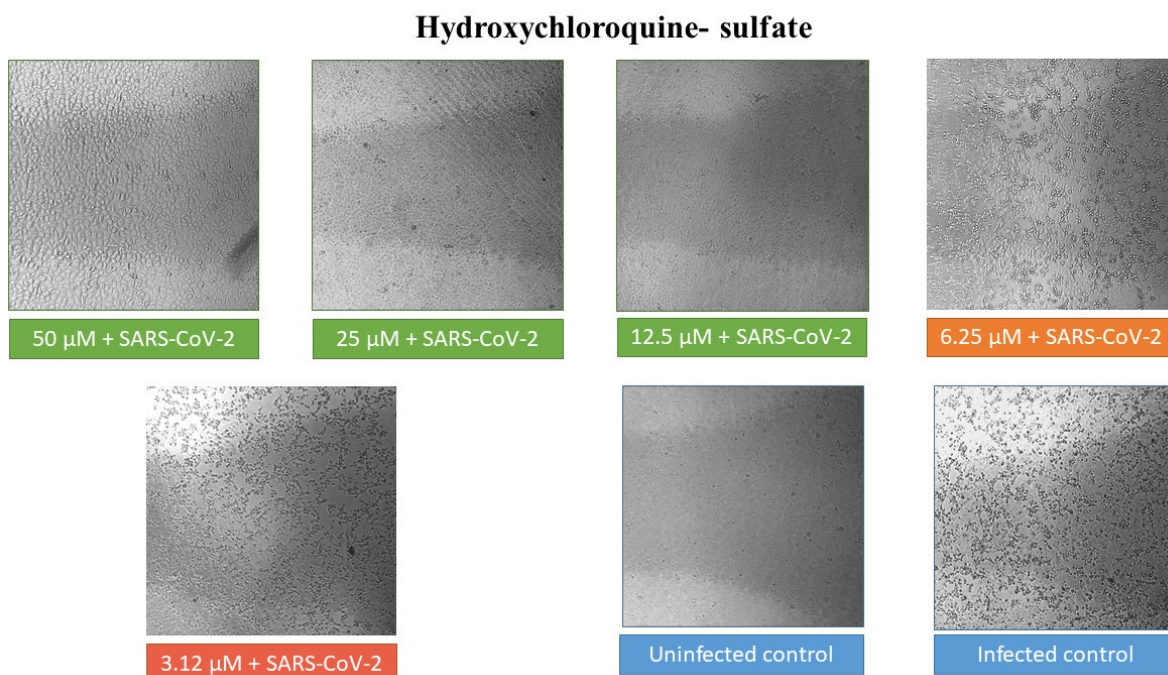


Figure 8. Microscopic evaluation of the HCQ antiviral effect. HCQ and SARS-CoV-2 were added to Vero E6 cells. After 30 minutes of infection, the virus-containing media was discarded, and cells were incubated for 48 hours in the presence of the compound. Signs of infection at higher concentrations were not detected by microscopic examination. Rounded cells and damaged monolayer were seen at low micromolar concentrations.

RNA was isolated from the cell culture supernatant, and the RdRp copy number of each sample was determined. IC₅₀ values were calculated for both compounds. Our results were within the range of the IC₅₀ values reported by other groups. In our screen, remdesivir had an IC₅₀ value of 2.4 μM. Other groups also reported low IC₅₀ values of remdesivir; for instance, Choi *et al.* also calculated an IC₅₀ of 2 μM in their screen [72]. IC₅₀ value of HCQ was 4.8 μM, which was in agreement with Liu *et al.*'s findings, which was 4.5 μM [73].

These results confirmed the reliability of our antiviral testing and evaluating method. Therefore, we were able to start to seek effective compounds with confidence.

4.1. *In vitro* screening of drugs for drug repositioning purposes

4.1.1. Rucaparib hinders the replication of SARS-CoV-2 *in vitro*

Rucaparib (Rubraca[®]) is an FDA/EMA-authorized poly-(ADP-ribose) polymerase (PARP) inhibitor. Currently, besides rucaparib, there are other PARP inhibitors in clinical use, olaparib (Lynparza[®]), niraparib (Zejula[®]) and talazoparib (Talzenna[®]). All four compounds are used in anti-cancer therapies, more precisely as maintenance therapy in patients with ovarian-,

fallopian tube-, and peritoneal cancer [74,75]. PARPs cleave nicotinamide adenine dinucleotide (NAD⁺), which results in the release of ADP-ribose. PARPs subsequently couple ADP-ribose units onto their acceptor proteins. PARP activity plays an important role in several cellular processes, for instance, cell death, immune responses, transcription, and translation. PARPs recognise DNA breaks and signals to the cell DNA repair enzymes [75,76]. The macrodomain of nsp3 can hydrolyse the ADP-ribose modifications of proteins, and by this, it can reverse the PARP-induced ADP-ribosylation; thus, it counteracts the host IFN-response [7,17]. In our study, we tested *in vitro* the repurposing potential of three registered PARP inhibitors, rucaparib, talazoparib and olaparib, for the treatment of COVID-19 [77]. First, we determined the concentration range in which the selected inhibitors do not induce cell death in Vero E6 cells. CellTiter-Glo[®] Luminescent Cell Viability Assay was used to assess the cytotoxicity. We found that all tested PARP inhibitors were safe to use below 40 μ M. In the case of rucaparib, we could determine the concentration where 50 % of the cells are viable (CC₅₀). In contrast, in the case of olaparib and talazoparib, the data were inadequate to obtain the CC₅₀ (Fig. 9).

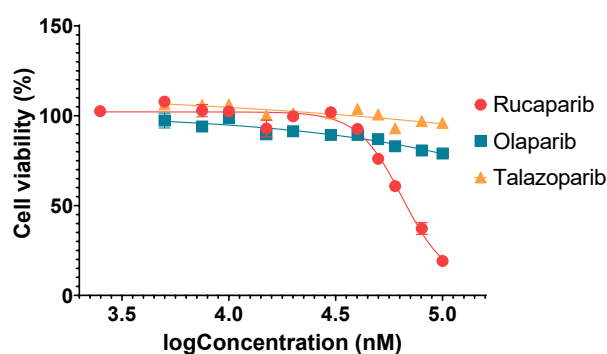


Figure 9. Cell viability of PARP inhibitor-treated Vero E6 cell. Vero E6 cells were more prone to the treatment with rucaparib than olaparib or talazoparib. Vero E6 cells are susceptible to rucaparib treatment with a CC₅₀ value of 64.8 μ M. CC₅₀ was calculated from three replicates and visualised using GraphPad Prism version 8.

After determining the non-toxic concentrations of the PARP inhibitors, the anti-SARS-CoV-2 effect was examined. Microscopic observation showed less CPE in the case of rucaparib-treated cells. To determine the IC₅₀ values of rucaparib, talazoparib and olaparib, the cells were treated with 40, 30, 20, 15, 10, 7.5, 5, and 2.5 μ M concentrations of the compounds above. Cells were treated with PARP inhibitors (rucaparib, talazoparib, and olaparib) in the indicated concentrations and immediately afterwards, SARS-CoV-2 B.1.5 at MOI 0.01 was added. Olaparib and talazoparib did not have antiviral activity, whereas rucaparib effectively blocked the viral replication (Fig. 6). As the indicated concentrations were not toxic to the Vero

E6 cells, the inhibition of the SARS-CoV-2 replication is unlikely to be due to the direct toxicity. The IC₅₀ of rucaparib was 27.5 μM, corresponding to ~8.7 mg/L. Following the recommended dose in a clinical setting (600 mg twice a day), rucaparib does not reach 8.7 mg/L in human serum. To achieve PARP inhibition in cancer patients, a lower concentration is sufficient. Supposedly, the measured antiviral activity relies on a different mechanism [78].

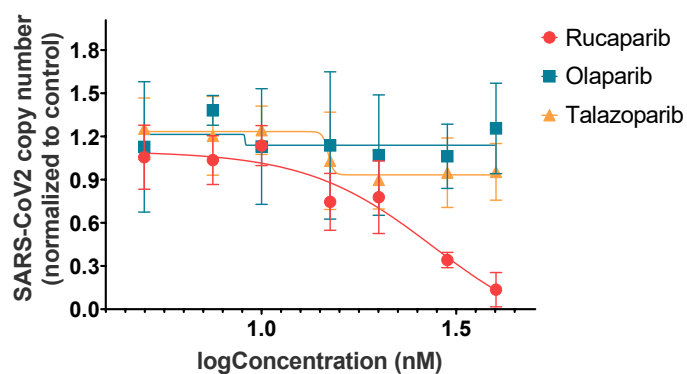


Figure 10. SARS-CoV-2 replication inhibition of PARP inhibitors in Vero E6 cell. In Vero E6 cells, the replication of the SARS-CoV-2 B.1.1.5 (MOI:0.01) variant was hindered by rucaparib (IC₅₀ 27.5 μM). While olaparib and talazoparib did not have antiviral effect. IC₅₀ was calculated and visualised using GraphPad Prism version 8. Data were obtained from three independent experiments.

To confirm that the *in vitro* antiviral activity of rucaparib is not limited to Vero E6, we tested its activity in A549 Dual™ hACE2-TMPRSS2 cells. Cell viability tests revealed that the human ACE2 and TMPRSS2 overexpressing A549 cells were more susceptible to rucaparib treatment than Vero E6. Rucaparib was toxic in concentrations above 10 μM. Ten μM rucaparib did not inhibit the SARS-CoV-2 replication in A549 Dual™ hACE2-TMPRSS2 cells (data not shown).

To sum up, in its current dosing regimen and route of administration, rucaparib might not be effective in inhibiting SARS-CoV-2 replication in clinical settings. However, it should be considered for further studies; for instance, by modulating the formulation of rucaparib-containing medicines to produce nasal sprays, a higher local concentration could be reached topically.

4.1.2. Anti-SARS-CoV-2 activity of azelastine hydrochloride

Konrat *et al.* applied a new computational approach to identify potent drugs against SARS-CoV-2 among clinically approved and commercially available compounds (repositioning or repurposing approach). The hypothesis was based on the fact that a pharmacon

can simultaneously interact with different targets that rewire biochemical pathways (poly-pharmacological hypothesis) [79]. Three experimentally verified compounds were selected to serve as the base of the pathway-based drug repositioning screen. The selected compounds (SSAA09E2, SSAA09E3 and HCQ) target the early steps of the viral replication, binding to ACE2, the fusion of the viral and host membrane, and the reduction of endosomal pH, respectively. Biochemical pathway profiles of drugs were then compared to the pathway activities of the selected compounds and pathways affected by SARS-CoV-2 infection [80]. Drugs that matched this predefined activity pattern and appeared in different pathway data were selected for further analysis [81]. We received eleven drugs to test *in vitro* against SARS-CoV-2. Our initial semi-quantitative screen was based on the cytopathic effect of the virus observed in drug-treated samples (Table 1).

| Drug class | Name | Concentration (μ M) | | | | |
|----------------|-------------------------|--------------------------|--------|--------|------|-------|
| | | 50 | 25 | 12.5 | 6.25 | 3.125 |
| Antiviral | maraviroc | +++ | +++ | +++ | ++++ | ++++ |
| Neuroactive | ziprasidone HCl | ++++ | ++++ | ++++ | ++++ | ++++ |
| Antidiabetic | tolbutamide | ++++ | ++++ | ++++ | ++++ | ++++ |
| Antineoplastic | axitinib | ++++ | ++++ | ++++ | ++++ | ++++ |
| Antiarrhythmic | esmolol | ++++ | ++++ | ++++ | ++++ | ++++ |
| | amiloride HCl | ++ | ++ | +++ | +++ | +++ |
| | telmisartan | + | + | + | ++ | ++ |
| | clevidipine butyrate | ++++ | ++++ | ++++ | ++++ | ++++ |
| | metoprolol | ++ | ++ | +++ | ++++ | ++++ |
| | losartan | ++ | +++ | +++ | +++ | ++++ |
| Antihistamine | azelastine HCl | Toxic | No CPE | No CPE | ++ | ++++ |

Table 1. CPE scores of the hits from *in silico* prediction. Vero E6 cells were treated with different drugs at five concentrations. The treatment and infection were done simultaneously (co-administration). Thirty minutes later, the drug and virus-containing cell culture media was replaced with media supplemented with the drug at the desired concentration. Forty-eight hours later, the cells were inspected under the microscope and the observed CPE was rated. ‘No CPE’ is comparable to uninfected control. +++++: CPE is as strong as in the infected control. +, ++, and +++++ scores represented the increasing extension of the host cell damage caused by the virus.

Amongst the identified drugs, antihistamine drugs have the potential to be used as anti-infectives too. Antihistamines could inhibit *in vitro* different bacteria spp. (e.g., *Staphylococcus aureus*, *Staphylococcus epidermidis*), *Candida* spp., protozoans (e.g., *Leishmania* spp., *Trypanosoma cruzi*) and viruses (e.g., IAV, EBOV, HCV) [82]. We selected azelastine HCl for further analysis as it is commercially available in nasal sprays (e.g., Astelin[®], Astepro[®], Allergodil[®], Pollival[®]). The benefit of using nasal sprays to contain SARS-CoV-2 spread relies on the fact, that nasal spray usage can form a physicochemical barrier and/or exert an antiviral effect at the first site of infection (nose and nasopharynx), thus impeding the disease progression to the lower airways. Azelastine HCl is a phthalazinone derivative that has histamine 1 receptor-blocking activity. It is used for the treatment of seasonal allergic rhinitis and nonallergic vasomotor rhinitis. Serious adverse event of azelastine treatment has not been reported yet. The most common side effect is a bitter taste, which has been mitigated by adding sucralose and sorbitol to the formula (Astepro[®]) [83,84]. The initial screen showed that 50 μM is toxic for Vero E6, so we studied the antiviral effect at $<50 \mu\text{M}$. Using an ATP-based cell viability assay, we found that 25 μM azelastine HCl did not cause damage in Vero E6 metabolism and morphological changes were also not detected. The antiviral effect of the azelastine HCl was measured at 25, 12.5, 6.25, 3.125, 1.6, 0.8, and 0.4 μM concentrations. We used two different settings to measure the azelastine HCl activity against SARS-CoV-2; co-administration and therapeutic. Azelastine HCl containing cell culture media was added to Vero E6 cells, and then the cells were infected with the virus (MOI: 0.01) (co-administration setting). Thirty minutes later, the media was replaced with azelastine HCl-containing cell culture media and incubated for 2 days. IC_{50} , in this case, was 2.2 μM . We aimed to determine the antiviral activity in the case when the cells are treated only after the initial infection (therapeutic setting). Thus, Vero E6 cells were first infected for 30 minutes, then the media was discarded, and the cells were incubated for two days with azelastine HCl-containing media. Azelastine HCl could exert antiviral activity, although it was a bit less effective; the IC_{50} value was 6.5 μM . The commercially available nasal sprays contain 3.6 mM and 2.5 mM of azelastine HCl (0.15% and 0.1% nasal sprays). Our promising results, together with the results of the researchers at the University of Innsbruck, were the starting point of a clinical trial (CARVIN) [81,85]. In preparation for the initiation of the clinical trial, we measured whether the nasal spray involved in the study (Pollival[®], Ursapharm, Germany) could interfere with the virus detection (inhibition of the RNA extraction or the PCR reaction). Undiluted and 100 fold diluted viral stocks were mixed with DMEM or Pollival[®] spray (1:1 ratio), and the virus was detected by

real-time PCR from the RNA extracted from those samples. PCR inhibition was not detected in any of the replicates (data are not shown due to confidentiality). We also compared the *in vitro* anti-SARS-CoV-2 activity of the commercial nasal spray, the pure active compound azelastine HCl, and the buffer of the nasal spray (used as its placebo in the clinical trial). The spray was used at the same concentrations as azelastine HCl. In all three independent experiments, we observed the same efficacy in the samples treated with the diluted nasal spray or azelastine HCl, while no anti-viral effect was observed with the buffer/placebo (data are not shown due to confidentiality).

Azelastine HCl has a potent anti-SARS-CoV-2 activity *in vitro*. It effectively blocks the replication even after the infection of the cells. Results from our screen were strengthened by our co-operators and served as the basis of a peer-reviewed publication and the initiation of clinical trials.

4.1.3. Methylene blue inhibits SARS-CoV-2 replication *in vitro*

Methylene blue potently inhibited the protein-protein interaction between human ACE2 and the receptor binding domain of SARS-CoV-2 spike protein in an ELISA assay [86]. Methylene blue was the first fully synthetic drug used in medicine. Since then, it has been applied in numerous medical procedures. It was used to treat malaria until it was replaced by chloroquine. Currently, it is used for treating methemoglobinemia, preventing urinary tract infections in elderly patients and for intraoperative visualisations. Furthermore, several studies have been made to explore its potential for treating different neurological disorders [87–89]. Methylene blue can be used as an antimicrobial agent as well. Methylene blue and light-activated methylene blue can inactivate lipid-enveloped viruses, such as HCV, West Nile virus (WNV), HIV-1, herpes simplex (HSV); and some non-enveloped viruses, for instance, human parvovirus B19 (hParvo-B19) and simian vacuolating virus 40 (SV40). Thus, it has been used to treat blood products before transfusion [90]. We aimed to confirm the observed inhibitory potential using infectious SARS-CoV-2. We found that methylene blue effectively blocks (IC₅₀ of 1.70 µM) the replication of SARS-CoV-2 (B.1.5) in Vero E6 cells [91]. Our results agrees with the findings of other groups that methylene blue has virucidal activity at low micromolar concentrations (IC₅₀ of 0.3-1.1 µM in Vero E6) [92–94]. Our study also confirms that methylene blue can be applied for the treatment of COVID-19. It would be more beneficial in low-income countries as well since methylene blue is a relatively cheap medication.

4.2. Potent hits from fragment-based drug design

Computer-aided drug development is a promising approach to discover drugs. When the three-dimensional structure of the targeted protein is unknown, pharmacophore-based approaches are relevant. Pharmacophores are steric and electronic features essential to ensure the molecular interactions between a biological target (e.g., enzyme, receptor) and its ligands that result in triggering or blocking a specific biological response [95,96]. Fragment-based drug design utilises screening of small polar compounds. Generally, the molecular weight of a fragment is <300 Da and has a limited number of pharmacophore features; thus, they are considered compact representatives of a much larger chemical space [97]. Bajusz *et al.* developed an *in silico* approach (SpotXplorer) that assembles fragments that bind to experimentally confirmed pharmacophores at the most preferred hotspots. The SpotXplorer technology can find from less than 100 molecules those that can bind robustly to a target. Screening the SpotXplorer fragment library resulted in one fragment hit against 3CLpro and five hits against the nsp3 macrodomain. Three fragment hits blocked SARS-CoV-2 (B.1.5) replication *in vitro* at high micromolar concentrations. EC₅₀ value of the 3CLpro binding, aryl-piperazine fragment was 304 μM. Amongst the hits of nsp3 macrodomain inhibitors, two proved to be effective with EC₅₀ values of 135.8 μM and 242.3 μM. These two potent fragments mimic the interaction between the nsp3 macrodomain and its natural ligand, the ADP-ribose. They are supposed to enclave into the adenosine-binding pocket of the macrodomain. The two fragments overlay in position with the adenine-proximal ribose sites of the ADP-ribose, and they provide growing and merging options for other compounds towards the distal ribose site. Further optimisation of those fragments would enable the generation of highly specific ligands. In conclusion, with the help of the SpotXplorer technology, we could identify potent lead molecules, that can serve as the basis of new drug candidates [96].

In conclusion, computer-aided drug discovery is a powerful approach to seek for active compounds. We confirmed the hits provided by a newly developed algorithm *in vitro*. The detected fragments could be lead molecules to develop novel direct-acting antivirals.

4.3. *In vitro* SARS-CoV-2 inhibition by glycopeptide antibiotic derivatives

Glycopeptide antibiotics (GPAs), such as teicoplanin and vancomycin, are one of the medications for treating life-threatening Gram-positive bacterial infections (*e.g.*, methicillin-resistant *Staphylococcus aureus*, *Enterococcus* spp.). Teicoplanin, vancomycin, eremomycin and ristocetin are obtained from natural sources, while oritavancin, telavancin and dalbavancin

are semi-synthetic derivatives [98,99]. Several drug development campaigns are focusing on modifying these antibiotics, due to the threat of multiresistant bacteria and, on the other hand, their broad-spectrum antimicrobial properties [100]. Antiviral activities of GPAs and their semi-synthetic derivatives have been described in the case of numerous viruses, such as IAV and influenza B virus (IBV), HIV-1, HIV-2, HCV, DENV, yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), WNV, ZIKV, HSV-1, HSV-2, RSV, CoVs (human coronavirus 229E (hCoV 229E), SARS-CoV, SARS-CoV-2, MERS-CoV) [100–104]. Based on our previous promising results with semi-synthetic teicoplanin and vancomycin antibiotic derivatives against the WNV [70] and the aforementioned antiviral properties of this antibiotic class, we tested different glycopeptide antibiotic derivatives. Firstly, we tested thirty-two derivatives at five concentrations (50, 25, 12.5, 6.25 and 3.125 μM) and monitored the CPE of the treated Vero E6 cells. Several hits were found in the CPE reduction assay [105,106]. One group of the potent SARS-CoV-2 inhibitors contained lipophilic apocarotenoids on teicoplanin, teicoplanin pseudo- ψ -aglycone and ristocetin aglycone. Even the apocarotenoids alone exerted antiviral activities. IC_{50} of bixin and β -apo-8'-carotenoic acid were 5.9 and 15 μM , respectively. Apocarotenoids are usually used in the food industry. Bixin is produced by *Bixa Orellana*, and crocetin naturally occurs in *Crocus sativus*. Both are biocompatible, non-toxic colourants. β -apo-8'-carotenoic acid is produced synthetically and used as a food colourant. Teicoplanin and bixin, teicoplanin ψ -aglycone and β -apo-8'-carotenoic acid conjugates had even more potent antiviral activity than bixin, β -apo-8'-carotenoic acid or the parent glycopeptide core. While crocetin monomethyl ester, teicoplanin ψ -aglycone, and ristocetin aglycone did not have antiviral effects at the examined concentrations (Table 2.).

| Glycopeptide antibiotic moiety | Apocarotenoid moiety | IC_{50} (μM) |
|--------------------------------|---------------------------------|------------------------------------|
| - | bixin | 5.9 |
| - | β -apo-8'-carotenoic acid | 15 |
| - | crocetin monomethyl ester | n.a. |
| teicoplanin ψ -aglycone | - | n.a. |
| teicoplanin | - | 5.6 |
| ristocetin aglycone | - | n.a. |

| | | |
|------------------------------|---------------------------------|-----|
| teicoplanin ψ -aglycone | bixin | 5.9 |
| teicoplanin ψ -aglycone | crocetin monomethyl ester | 5.2 |
| teicoplanin ψ -aglycone | β -apo-8'-carotenoic acid | 4.4 |
| teicoplanin | bixin | 1.8 |
| ristocetin aglycone | bixin | 6.7 |

Table 2: IC_{50} values of glycopeptide antibiotic and apocarotenoid conjugates. Anti-SARS-CoV-2 activity in Vero E6 cells of glycopeptide antibiotic derivatives. Cells were infected with MOI 0.01 B.1.5 SARS-CoV-2 and were treated with the compounds at different concentrations. IC_{50} values were calculated with GraphPad Prism 8 software from three replicates. Abbreviations: -n.a. not active.

Glycopeptide antibiotics bearing different hydrophobic, lipo- and hydrophobic perfluoroalkyl chain or guanidino groups were tested in CPE reduction. Hydrophilic and/or lipophilic side chains inhibited SARS-CoV-2 replication in the CPE reduction assay (Table 3) [106].

| | 50 μ M | 25 μ M | 12.5 μ M | 6.25 μ M | 3.125 μ M |
|--|------------|------------|--------------|--------------|---------------|
| Teicoplanin ψ -aglycone + Triazole-TEG-glucose-dioctyl | No CPE | + | +++ | ++++ | ++++ |
| Teicoplanin ψ -aglycone + guanidine | + | + | ++ | +++ | ++++ |
| Teicoplanin ψ -aglycone + Triazole-TEG-perfluorooctyl | + | +++ | +++ | +++ | +++ |
| Teicoplanin ψ -aglycone + Triazole-EG-perfluorobutyl | No CPE | + | ++ | +++ | ++++ |
| Teicoplanin ψ -aglycone + maleimide-dihexyl | + | ++ | +++ | ++++ | ++++ |
| Teicoplanin ψ -aglycone + Triazole-EG-perfluorooctyl | + | ++ | ++++ | ++++ | ++++ |
| Teicoplanin ψ -aglycone + amide-perfluorooctyl | Toxic | No CPE | + | +++ | +++ |

| | | | | | |
|---|-------|--------|------|------|------|
| Teicoplanin ψ -aglycone + amide-perfluorhexyl | Toxic | No CPE | + | +++ | ++++ |
| Teicoplanin ψ -aglycone + triazole-perfluorhexyl | Toxic | No CPE | ++++ | ++++ | ++++ |
| Teicoplanin ψ -aglycone + tri-guanidine | Toxic | +++ | +++ | ++++ | ++++ |

Table 3. CPE scores of teicoplanin ψ -aglycone derivatives. Cells were inspected with an inverted light microscope, and the CPE of the treated samples was compared to the infected control samples. 'No CPE' is comparable to uninfected control. +++++: CPE is as strong as in the infected control. +, ++, and +++++ scores represented the increasing extension of the host cell damage caused by the virus. Abbreviations: TEG-tetraethylene-glycol, EG-ethylene-glycol, CPE-cytopathogenic effect.

Antiviral activities of glycopeptide antibiotics and their derivatives have been studied extensively to date. Our group has a long-standing cooperation with the Department of Pharmaceutical Chemistry at the University of Debrecen. We were seeking compounds with potent anti-SARS-CoV-2 activity. We found effective compounds amongst teicoplanin antibiotic derivatives equipped with different apocarotenoid side chains or perfluoroalkyl-or guanidine moieties.

5. Discussion

The response of the scientific community to the COVID-19 pandemic was unprecedented. Within a few months, peer-reviewed journals and ArXiv preprint servers were full to the brim with reports about the characteristics of the disease and the virus itself. Numerous studies were conducted to identify active drugs, clinical candidates, and lead molecules that are active against SARS-CoV-2 or cytokine storm triggered by the infection. High-throughput screenings using pseudoviruses, viral mini replicons or SARS-CoV-2 in high containment facilities, enzyme interaction assays, and modern computational techniques (e.g., molecular docking, machine learning, ligand-based strategy etc.) were utilised to provide a large pool of potential antiviral candidates [38,107]. More than 500 compounds were sent to our research group for anti-SARS-CoV-2 testing. Amongst these compounds, there were numerous pharmaceutical drugs (prescription, over-the-counter), natural products, chemical fragments, newly synthesised nucleoside analogues, semi-synthetic glycopeptide antibiotic derivatives and so on. We cooperated with numerous excellent biologists, chemists, pharmacologists and researchers from other disciplines. The intensive research on SARS-CoV-2 and the more rapid publication processes helped to gain and distribute knowledge about the newly emerged virus quicker and more thoroughly.

As SARS-CoV-2 is classified as a BSL-3 agent, any experiment involving work with the infective viral particle must be performed in at least a BSL-3 laboratory. There are numerous valuable tools for the effective and safe studying of virulent or lethal viruses without the need for high containment facilities. The more common is the use of pseudotyped viruses. Using a cell-free ELISA assay, along with a commercially available SARS-CoV-2 pseudovirus assay, methylene blue was identified as a potential direct-acting antiviral. The assays used in this study focused on small-molecule protein-protein interaction inhibitors. In the case of SARS-CoV-2, one of the most important protein-protein interactions occurs at the viral attachment and entry. Methylene blue dose-dependently inhibited the interaction between the SARS-CoV-2 spike and ACE2 protein (IC_{50} of 3.5 μ M) [86]. Methylene blue is an inexpensive and clinically used, versatile organic dye. It is used for the treatment of methemoglobinemia. It is also used to prevent urinary tract infection in elderly patients; during operations, nerves, nerve tissues and endocrine glands are also stained in some instances to aid intraoperative visualisations, and it also ameliorates vasoplegic syndrome. It is also used to inactivate pathogens in blood products before transfusion [86,87,89,90]. We aimed to confirm the results obtained from the screen above. We found that methylene blue can inhibit the replication of SARS-CoV-2 in Vero E6

cells at low micromolar concentrations (IC_{50} of 1.7 μ M). Our results agree with other studies that found methylene blue effective in inhibiting SARS-CoV-2 at low micromolar concentrations [91–93]. These concentrations, which exert antiviral activity in the different screens, are within the range of the clinically achieved blood concentrations. Peak blood concentration (19 μ M) was reached after administering 500 mg *per os*. Once-daily oral administration can be beneficial for the treatment of COVID-19 patients. Moreover, methylene blue can reduce the oxidised form of hemoglobin as a result, it increases its oxygen-binding capacity that helps the oxygen delivery to tissues. COVID-19 patients often exhibit hypoxemia [91]. To date, there are some data regarding methylene blue effectivity in the management of COVID-19 patients. In a phase I study, methylene blue-vitamin C–N-acetyl cysteine treatment was applied in 5 patients at the intensive care unit. Four out of five patients responded well to this treatment [108]. Results from a phase III trial have also been reported. Two hundred twenty-three patients with severe COVID-19 were enrolled in this study. The hospital stay was significantly shortened in the standard care plus methylene blue treated group. Mortality was also lower in the methylene blue group [109]. There are other registered, completed, or active trials. More results would be beneficial to state truly, that methylene blue should be used to treat COVID-19.

Studies showed that the macrodomain of SARS-CoV-2 and other viruses can reverse ADP-ribosylation, which can impede the innate immune response of the host [17,76,110]. Furthermore, ADP-ribosylation of capsid proteins of CoVs has been observed [111]. SARS-CoV-2 infection can also dysregulate the different PARP gene expressions and thus challenging the NAD metabolism [112]. These overlaps between the SARS-CoV-2 infection and the poly-ADP ribosylation and *in silico* studies suggesting PARP-inhibitors for the treatment of COVID-19 lead us to test the repurposing potential of registered PARP inhibitors [113,114]. In addition, patients receiving PARP inhibitors as part of their cancer therapy responded worse to SARS-CoV-2 vaccination than those not treated with PARP inhibitors [115]. Based on these reports, we selected four antineoplastic agents, olaparib (Lynparza[®]), niraparib (Zejula[®]), talazoparib (Talzenna[®]), and rucaparib (Rubraca[®]) to test their activity against SARS-CoV-2. We found that rucaparib at supra-pharmacological concentrations can inhibit the replication of SARS-CoV-2 *in vitro*. In cooperation with other groups, we aimed to identify the mechanisms behind the observed antiviral effect. Using fluorescently labelled pseudoviruses bearing spike proteins of different SARS-CoV-2 variants of concerns, saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy and *in silico* modelling, we proposed that rucaparib

binds to the ACE-2 binding site of the spike protein thus counteracting the viral entry [77]. Although the chemical interaction detected between rucaparib, and the spike protein is not very potent. It can explain the relatively high IC₅₀ value. The steady-state levels of rucaparib in the serum during cancer therapy do not reach these levels. Therefore, rucaparib treatment at the currently applied dosage regimens presumably would not exert antiviral activity. On the other hand, PARP1 activation is considered proinflammatory in Th1 and Th2-mediated immune responses [116]. Our study also found that the anti-inflammatory potential of rucaparib was comparable to dexamethasone. Learning from the *in vitro* studies focusing on stenoparib, an investigational, orally available small molecule PARP inhibitor, combinational therapy can also be advantageous to successfully treat COVID-19 patients with a PARP inhibitor. However, to be certain more studies focusing on the toxicity, penetration and pharmacokinetics need to be conducted [117,118]. Consequently, rucaparib can be a promising candidate for further drug development campaigns. Most of the amino acids found in the binding pocket of rucaparib are conserved among SARS-CoV-2 variants, suggesting the effectivity against newly emerged variants as well. Higher local concentrations can be achieved by applying rucaparib in the aerosol formulation. By this, dual effects (antiviral and immunomodulatory) in the lungs can be achieved as well.

The usage of nasal sprays can be beneficial for several reasons. By using a spray, a physicochemical barrier can be formed and consequently, viral attachment can be obstructed. On the other hand, nasal sprays bearing antiviral activity can contribute to eliminate the virus from the upper respiratory tract. As a result, the progression of the disease to the lower airways and the airborne transmission of the virus might be prevented. A few commercially available nasal drugs have already been tested in clinical trials against COVID-19. Patients or healthcare workers were treated with iota-carrageenan or nitric oxide, or povidone-iodine-containing nasal sprays [119–122]. Results of these trials showed promising results for preventing the infection or developing moderate or severe disease. Our project with Cebina GmbH revealed that azelastine HCl could inhibit SARS-CoV-2 *in vitro* [81]. Over-the-counter azelastine HCl-containing nasal sprays and eye drops are used to treat hay fever and allergy symptoms (such as stuffy or runny nose, itching, sneezing, and post-nasal drip). The safety profile is well characterised even in chronic administration, and children above the age of 6 years can be treated with it without the risk of severe adverse events [83]. Moreover, azelastine can stabilise mast cells and inhibit the production of pro-inflammatory cytokines and leukotrienes [123–125]. Mast cells produce cytokines that lead to lung pathologies in SARS-CoV-2 [126–128].

Thus, azelastine HCl can be a potent drug for treating cytokine storm induced by SARS-CoV-2 infection. Our study demonstrated that different SARS-CoV-2 variants are inhibited efficiently *in vitro* [81]. This activity can be explained by the high affinity of azelastine HCl to ACE2. More significantly, it binds to the Lys353, which has been identified as one of the hotspots of spike-ACE2 binding. Accordingly, azelastine may impede viral entry to ACE2-expressing cells [129]. The SARS-CoV-2 inhibitory concentrations were lower than those in the commercially available azelastine HCl-containing medications. Our study indicated that topical administration of azelastine HCl can be effective for intercepting SARS-CoV-2 infection in the nasopharyngeal area. An effective dose needed for antiviral activity could be achieved at the target site (nose) without unwanted systemic adverse events [81]. Based on our results, a phase II clinical trial was conducted to repurpose Pollival[®], an azelastine HCl-containing nasal spray. In the CARVIN trial, 90 non-hospitalised SARS-CoV2-positive patients were enrolled. Patients were treated either with placebo (hypromellose, disodium edetate, citric acid, disodium phosphate dodecahydrate, sodium chloride and purified water), 0.1 % or 0.02 % azelastine HCl (placebo supplemented with 1 mg/ml or 0.2 mg/ml azelastine HCl, respectively) containing nasal spray. 0.02% azelastine HCl spray was prepared and studied because the *in vitro* results indicated that antiviral activity can be achieved at low micromolar concentrations. Patients were treated for eleven days; one puff was administered in each nostril 3 times a day. Viral load was monitored with quantitative real-time PCR, specific to ORF1 a/b and E genes. This pilot proof-of-concept study showed that azelastine HCl-containing nasal spray could reduce viral replication without causing severe adverse events [85]. Mathematical modelling predicts that using the nasal spray 5 times per day and applying it orally can be beneficial [130]. The available data is very promising, and further phase III. studies are ongoing to fully elicit the potential of azelastine HCl-containing medications to treat COVID-19.

A pathway-based computational approach led to the discovery of the potential polypharmacological properties of azelastine HCl. Computer-aided rational drug design is a rapidly expanding field. *In silico* approaches can curtail the time and cost of drug discovery, lead optimisation and assist in raising the quality of drug candidates, thus decreasing the failure rate at the clinical phase. Bajusz *et al.* developed a new algorithm (SpotXplorer), to identify the Achilles heel, the most important targetable area of disease-causing proteins. Important targetable areas (so-called hotspots) can also be found on mammalian cells and pathogens [95]. Accurate knowledge of these hotspots can help target specific mechanisms of infectious diseases. Utilising the SpotXplorer technology, fragment libraries were generated from small

molecules that could cover the pharmacophores of SARS-CoV-2 main protease and nsp3 macrodomain. The identified small molecules were tested against SARS-CoV-2 *in vitro*. The results obtained from the *in vitro* screen could confirm the reliability of the fragment-based drug discovery approach and provide lead molecules for further drug development campaigns. Fragments, for example, could be merged with other fragments that bind to different targetable regions (hotspots) to potentiate their inhibitory activity [96].

Phenotypic drug discovery has led to the discovery of many drugs or their antecedents. It is based on the observations of the therapeutic effect on disease phenotypes of a compound in disease-relevant biological systems (cell-based assays, *in vivo* models or even in humans). For instance, the HCV replicon phenotypic screen helped the discovery of daclatasvir, a direct-acting antiviral [131]. Favipiravir was also found by an extensive phenotypic screen led by Toyama Chemical Co., Ltd to find anti-influenza agents [56]. Glycopeptide antibiotics such as teicoplanin, dalbavancin, oritavancin, vancomycin, and telavancin are used to treat Gram-positive bacterial infections [99]. Several studies showed that glycopeptide antibiotics and their derivatives have antiviral activities against a wide range of viruses [100]. Derivatives of such antibiotics could block the entry of different retroviruses [132,133]. In the case of the HCV, semi-synthetic teicoplanin derivatives blocked the viral replication *in vitro* [134]. Moreover, teicoplanin therapy decreased the HCV load in a patient [101]. Antiviral screens utilising pseudoviruses (EBOV, MERS-CoV, SARS-CoV) also showed promising results [103,135]. Influenza viruses were also effectively blocked by different analogues of glycopeptide antibiotics [136,137]. Teicoplanin or modified teicoplanin has been proven to block flaviviral replication in cell culture [70,102]. Viruses related to SARS-CoV-2 were also inhibited by such compounds [103,138]. We also tested different glycopeptide antibiotic derivatives, synthesised by Prof. Anikó Borbás's group. Teicoplanin ψ -aglycone, ristocetin aglycone and teicoplanin conjugated with apocarotenoids showed a similar antiviral effect in Vero E6 cells. The most robust antiviral effect against SARS-CoV-2 was exerted by the bixin conjugate of teicoplanin. Our results showed that lipophilic moieties are beneficial to have the antiviral effects of glycopeptide derivatives. Interestingly, two apocarotenoids, bixin and β -apo-8'-carotenoic acid, also blocked the replication of SARS-CoV-2 *in vitro*. Bixin and the ethyl ester of β -apo-8'-carotenoic acid are approved, widely used, cheap, non-toxic, and natural colourants (yellow/orange pigments) used as food additives. Moreover, while exhibiting antiviral properties, the apocarotenoid conjugates also had antibacterial activities even against vancomycin-resistant *Enterococcus faecalis* strains [105]. In our other study, derivatives

bearing different synthetic modifications (double lipophilic tail, perfluoroalkyl chains, and guanidine group) also effectively increased the activity of the parent teicoplanin antibiotic against both SARS-CoV-2 and resistant bacteria [106]. The mechanism behind the antiviral activity of the derivatives we tested could be very diverse. It has been previously demonstrated that teicoplanin can block the SARS-CoV and MERS-CoV pseudovirus entry by impeding the activity of cathepsin L in a dose-dependent manner [103]. It has been reported that during SARS-CoV-2 entry, cathepsin L and B play an important role in the proteolytic priming of the S protein [139]. In addition, teicoplanin exhibited 3CLPro inhibition in biophysical assays [140]. The cathepsin L blocking activity, the exopeptidase activity of cathepsin B, and the protease activity of the viral main protease (Mpro) were also examined in the presence of the compounds. We found that the conjugates of teicoplanin ψ -aglycone with apocarotenoids have a combined mechanism of action based on cathepsin and 3CLPro inhibition. At the same time, bixin could only inhibit the cathepsin L enzymatic activity. Low-mode molecular docking analysis revealed that binding to cathepsins B and L was probably due to the apocarotenoid chains. At the same time, the main structural units mediate the binding to the main protease [106]. Fu *et al.* also demonstrated that teicoplanin could suppress the enzymatic activity of cathepsin L. Moreover, they also examined the teicoplanin antiviral activity *in vivo* as well, using mice expressing human ACE2. They found that pre-treatment of teicoplanin prevented the infection of mice [141]. Interestingly, a recent study showed that teicoplanin could act directly against SARS-CoV-2 too. Ma *et al.* tested different teicoplanin derivatives and found they can bind to the spike protein ACE2 binding residues. Their study involved pseudoviruses expressing spike protein with D614G mutation or presented at delta and omicron variants [142]. The antiviral properties, the threat of bacterial co-infections, the oral administration and the known pharmacokinetics led to testing of teicoplanin therapy in COVID-19 patients [143–145]. Treatment of COVID-19 patients admitted to the intensive care unit for severe respiratory complications was complemented with teicoplanin. No significant difference was observed in viral clearance and length of stay in the ICU. The authors proposed that teicoplanin treatment would be more beneficial at the early stages of infection as viral replication is more dominant [146]. While in another study, mortality was significantly lower in hospitalised patients in the teicoplanin arm [147]. Further studies are needed to evaluate the efficacy of teicoplanin for treating COVID-19. Based on the proposed mechanisms of antiviral activity of teicoplanin. It would be beneficial to start the treatment as soon as COVID-19 is diagnosed. In this scenario, inhibition of viral entry by teicoplanin would be more relevant. On the other hand, drugs bearing guanidine moieties (such as proguanil, metformin, and camostat mesylate were also reported to

be effective against SARS-CoV-2. Camostat mesylate and metformin have also been tested in clinical trials [58,148]. To date, the available data from the trial are insufficient to conclude the usability of those drugs for COVID-19 treatment. Although a derivative of metformin showed promising results [149]. More studies involving various derivatives with guanidine groups would be advantageous to develop good clinical candidates. Especially, those that showed dual antimicrobial activity could be good lead molecules.

To date, eight medicines are authorised in the European Union to treat COVID-19. Evusheld[®] (tixagevimab, cilgavimab), Regkirona[®] (regdanvimab), Ronapreve[®] (casirivimab, imdevimab) and Xevudy[®] (sotrovimab) target the spike protein of SARS-CoV-2. Kineret[®] (anakinra) and RoActemra[®] (tocilizumab) are prescribed for treating, for instance, rheumatoid arthritis or cryopyrin-associated periodic syndromes. Anakinra is an interleukin-1 receptor antagonist, and tocilizumab blocks the IL-6 receptor. Both proved to improve the conditions of severely ill COVID-19 patients. Veklury[®] (remdesivir) is also used to treat hospitalised COVID-19 patients. The only EMA-authorised medication that can be orally administered is Paxlovid[™] (nirmatrelvir, ritonavir). It can be prescribed for those SARS-CoV-2 patients who do not require supplemental oxygen and has an increased risk of developing severe symptoms [61]. In Hungary, favipiravir can also be prescribed for the treatment of mild COVID-19. However, studies are still inconclusive regarding its effectiveness [56]. Learning from previous viral disease outbreaks, it is beneficial to have consistent antiviral research even after the disease has been passed [107]. One of the biggest concerns is the development of antiviral resistance. Drug resistance has evolved in the case of HCV, HIV, HBV, IAV and IBV. *In vitro* and *in silico* studies have pointed out that the appearance of drug-resistant SARS-CoV-2 variants is reasonable [150–153]. Although luckily, it has not been reported yet. However, continuous scientific interest and robust financial investment can yield the desired fruit and can aid in preventing the lasting economic, social, and physical damage caused by an outbreak. Constant antiviral research is essential to reach this goal and to make it more effective collaborations are essential. It would also be advantageous to focus on multitarget drugs (polypharmacology) instead of one-target therapeutic agents. Multi-target approaches (drugs bearing several modes of action or combinational therapies) offer a safer therapeutic solution. In one way, developing resistance is more complicated in combinational therapies. On the other hand, simultaneous modulation of different targets can exert therapeutic effects at lower concentrations; thus, side effects can be minimised. Gaining insights into the mode of action of

a therapeutic agent is also essential to develop high-quality clinical candidates and designing potent treatments.

6. Summary

In 2019 a new coronavirus, SARS-CoV-2 emerged in China and then spread all around the globe within a brief time. COVID-19 put a considerable burden on the healthcare system of every country. The number of reported positive cases grew minute by minute. Many patients were transferred to the intensive care unit. Fatalities were taking on alarming proportions. To contain the viral transmission, governments needed to apply desperate measures collectively known as lockdowns. Social contacts were curtailed: sporting and cultural events were cancelled, restaurants were closed, if it was feasible remote work was ordered, kids needed to study at home, and curfew came into force. Lockdowns did help to shrink the infection rate but also took a heavy toll on the economy of every country. Scientists and medical professionals worked day and night to find the best solutions to halt COVID-19. An unprecedented partnership was formed between academia, industry and government.

During my PhD project, my main aim was to identify compounds with potent inhibitory activity against SARS-CoV-2 *in vitro*. We received more than 500 compounds to conduct *in vitro* antiviral tests. Amongst them, there were numerous prescription or over-the-counter drugs used in human or veterinary health care. We tested newly synthesised synthetic, semi-synthetic compounds and natural compounds. Moreover, we evaluated the activities of smaller building blocks used to modify parent compounds.

We proved that rucaparib, an antineoplastic agent, can hinder the replication of SARS-CoV-2 in cell culture. To the best of our knowledge, we reported first this observation. According to the results of our collaborators, the rucaparib can interact with the spike protein and inhibits entry. However, we presume that direct-antiviral activity would not be possible by the currently applied dosing regimen and administration route. On the other hand, for COVID-19 treatment, the immunomodulatory effect of rucaparib should not be ruled out as well. We proposed that after further modifications, rucaparib, or its derivative could be a potent medication to prevent SARS-CoV-2 infection.

We confirmed the repurposing potential of methylene blue as well. Our results align with the results of other studies. Even though we were not the first group to report the inhibitory

effect of methylene blue on the infectious SARS-CoV-2, our result further strengthened the mode-of-action study of our collaborator.

Computer-aided drug discovery and rational drug design became a very powerful approach to identify promising compounds for drug development campaigns. We also took part in projects that utilised *in silico* analyses. We joined Cebina GmbH to validate the results of a pathway-based analysis. Through our *in vitro* antiviral assay, we found a very effective compound, azelastine HCl. As it is found in over-the-counter nasal sprays and eye drops with an excellent safety profile, we further tested its potency. Based on our results, a phase II clinical trial has also been conducted under the coordination of Cebina GmbH and Ursapharm GmbH. We have been included in another study utilising a computational analysis to seek directly acting antiviral compounds. The novel SpotXplorer technology can identify the Achilles heel, the most important targetable area (hotspot) of disease-causing proteins. In principle, many molecules can bind to the hotspots of the proteins, but only a few molecules can bind effectively. The SpotXplorer technology assembles fragment libraries to maximise the chances of finding such molecules. Utilising the SpotXplorer fragment library of 96 compounds, we could identify fragment-sized hit compounds that are specific to the nsp3 macrodomain and 3CLpro. This technology can open new horizons in the development of truly effective medicines.

Our research group had already studied the antiviral activities of newly synthesised derivatives of glycopeptide antibiotics before SARS-CoV-2 emerged. Literature data suggested that such compounds can also be effective against the recently emerged virus. Potent SARS-CoV-2 inhibitors were found amongst those derivatives. Teicoplanin and teicoplanin ψ -aglycon were the parent glycopeptide of most of the inhibitors. Interestingly, widely used, cheap food additives that served as modifying groups on the parent compounds showed antiviral activity too. Furthermore, derivatives containing guanidino-, or perfluoroalkyl chains proved to be effective compounds as well against SARS-CoV-2 *in vitro*.

To further characterise the potential of those compounds *in vitro*, more experiments would have been advantageous. CC_{50} should have been determined in more cases, and subsequently, the selectivity index would have been resolved. Furthermore, infecting the cells with a higher number of infectious viral particles and with other variants of concern could help us to determine the spectrum of the antiviral activity. Last but not least, by involving more types of *in vitro* models, more information would have been provided to the translational medical science experts. A549 Dual™ hACE2-TMPRSS2 cells and 3D models cultured at the air-liquid

interface should have been utilised more often. Moreover, collaboration with groups with expertise in working with organoids or human organs-on-chips technologies would have been prosperous. These *in vitro* models would have provided more potent evidence to invest in developing the described compounds.

In summary, we built fruitful collaborations with both academic and industrial partners. We gained more experience in antiviral testing. Our results provided information for further drug development campaigns. We identified a good clinical candidate tested already in clinical settings (CARVIN trial) with promising results. Finally, more compounds become available for our research group, initiating further undergraduate and graduate student projects possible.

7. Összefoglaló

2019-ben egy új típusú koronavírus, a SARS-CoV-2 jelent meg Kínában, majd rövid időn belül az egész világon elterjedt. Az egészségügyi ellátórendszer világszerte, eddig nem tapasztalt mértékben leterhelődött a COVID-19 betegek számának robbanásszerű növekedése következtében. Számos koronavírusos beteget kellett az intenzív osztályon kezelni, a halálesetek riasztó méreteket öltöttek. A vírus terjedésének megfékezése érdekében az országfőknek kétségbeesett intézkedéseket kellett alkalmazniuk. A lakosság mindennapi életét korlátozó intézkedéseket vezettek be: betiltották a sport-és kulturális rendezvényeket, bezártak az éttermeket, ha lehetett távmunkát rendeltek el, a gyerekeknek otthon kellett tanulniuk, életbe lépett a kijárási tilalom. A bevezett korlátozó intézkedések segítettek csökkenteni a fertőzési arányt, de súlyos károkat okoztak a gazdaságában. A kutatók és az egészségügyi szakemberek éjjel-nappal dolgoztak, hogy megtalálják a legjobb megoldásokat a COVID-19 megállítására. Sok esetben, példátlan összefogás jött létre az akadémia, az ipar és az országok vezetése között.

Doktori munkám során fő célom volt olyan vegyületek azonosítása, amelyek *in vitro* kifejezett gátló hatással rendelkeznek a COVID-19 kórokozója, a SARS-CoV-2 ellen. Több mint 500 vegyület érkezett kutatócsoportunkhoz, hogy azok *in vitro* antivirális tesztjeit elvégezzük. Ezek között számos vényköteles vagy vény nélkül kapható gyógyszer hatóanyaga is volt, melyet vagy a köz-, vagy állategészségügyben alkalmaznak. Újonnan szintetizált szintetikus, félszintetikus és természetes eredetű vegyületeket is vizsgáltunk. Ezen túlmenően vizsgáltuk, bizonyos alapvegyületek származékainak előállításánál felhasznált származékoló vegyületek antivirális aktivitását is.

Bizonyítottuk, hogy a rukaparib, ami egy tumorellenes gyógyszer hatóanyaga, gátolja a SARS-CoV-2 replikációját Vero E6 sejtekben. Legjobb tudomásunk szerint mi írtuk le ezt a megfigyelést először. Ebben a kutatásban részt vevő további kutatók eredményei alapján, a rukaparib kölcsönhatásba léphet a tüskefehérjével és ezáltal képes gátolni a vírus bejutását. Feltételezzük azonban, hogy jelenleg, a klinikumban alkalmazott kezelési módszerrel, nem lenne elérhető, közvetlenül a vírusra gyakorolt gátló hatás. Továbbá, a COVID-19 kezelés esetében a rukaparib immunrendszerre kifejtett szabályozó hatását is érdemes lenne kiaknázni. Feltételezzük, hogy további kémiai módosítások után, a rukaparib vagy származéka hatékony gyógyszer hatóanyag lehet a SARS-CoV-2 fertőzés megelőzésére.

Megerősítettük a metilénkék SARS-CoV-2 gátló hatását is. Eredményeink összhangban vannak más tanulmányok eredményeivel. Annak ellenére, hogy nem mi voltunk az első

kutatócsoport, aki beszámolt a metilénkék SARS-CoV-2 gátló hatásáról, munkánk erősítette a velünk kooperáló kollégák méréseit.

A számítógépes analízist alkalmazó gyógyszerfejlesztés és a racionális gyógyszertervezés nagyon hatékony eljárás az ígéretes vegyületek azonosításában. Olyan kutatásokban is részt vettünk, amelyek *in silico* elemzéseket alkalmaztak. Eredményes munkakapcsolatot alakítottunk ki a Cebina Kft-vel. Validáltuk a sejtes válaszokat alapul vevő számítógépes analízisük során talált hatóanyagok SARS-CoV-2 elleni hatékonyságát. *In vitro* antivirális vizsgálatunk segítségével az azelasztin-HCl erős vírus gátló hatása tűnt ki. Mivel ezen hatóanyag vény nélkül kapható orrsprayben és szemcseppben megtalálható, biztonságosan alkalmazható, tovább teszteltük a hatékonyságát. Eredményeink alapján a Cebina Kft. és az Ursapharm Kft. koordinálásával II. fázisú klinikai vizsgálat is lezajlott. Csatlakoztunk egy másik kutatáshoz is, amely számítógépes elemzést alkalmazott a vírúsgátló vegyületek felkutatására. Az új SpotXplorer technológia képes azonosítani a betegséget okozó fehérjék Achilles-ínját, a legfontosabb célterületét (hotspot). Sok molekula tud kötődni a fehérjék hotspotjaihoz, de csak néhány molekula képes hatékonyan kötődni. A 96 vegyületből álló SpotXplorer fragmentskönyvtárat felhasználva sikerült azonosítani az nsp3 makrodoménre és 3CLpro-ra specifikus, kisméretű vegyületeket. Ez a technológia új távlatokat nyithat meg az igazán hatékony gyógyszerek fejlesztésében.

Kutatócsoportunk már a SARS-CoV-2 megjelenése előtt tanulmányozta a glikopeptid antibiotikum származékok vírusellenes hatását. Irodalmi adatok arra utalnak, hogy az ilyen típusú vegyületek az újonnan megjelent vírusok ellen is hatásosak lehetnek. E származékok között erős SARS-CoV-2 inhibitorokat találtunk. Az SARS-CoV-2-t gátló származékok többségének kiindulási glikopeptidje a teikoplanin és a teikoplanin ψ -aglikon volt. Érdekes módon, vírúsgátló hatást mutattak a széles körben használt, olcsó élelmiszer-adalékok, amelyek származékoló szerként szolgáltak az glikopeptid antibiotikum alapvázon. Továbbá, a guanidino-vagy perfluoralkilláncot tartalmazó származékok is hatékonyak bizonyultak a SARS-CoV-2 ellen *in vitro*.

Megemlítendő igaz, hogy az értekezésemben tárgyalt vegyületek *in vitro* hatékonyságának még pontosabb jellemzésére további kísérleti módszerek bevonása előnyös lett volna. A CC_{50} értéket több esetben meg lehetett volna határozni, és ezt követően a szelektivitási indexet is érdemes lett volna meghatározni. Továbbá, ha a sejteket nagyobb mennyiségű fertőző vírussal, illetve további variánsokkal is fertőztük volna, akkor az segíthetett volna jobban körülírni a vegyületek vírusellenes hatásának spektrumát. Végül, de nem utolsósorban, több munkánk

során is bevonhattunk volna más típusú sejtes modellt, mint például az A549 Dual™ hACE2-TMPRSS2 sejtek és 3D sejt kultúrák. Ezeknek az *in vitro* modelleknek a felhasználása erőteljesebb bizonyítékot szolgáltatna a vizsgált vegyületek fejlesztésébe való befektetéshez.

Összefoglalva, gyümölcsöző együttműködések építettünk ki tudományos és ipari partnerekkel. Több tapasztalatot szereztünk az antivirális tesztelésben. Eredményeink alapul szolgálhatnak további gyógyszerfejlesztésekhez. Sikerült azonosítanunk egy olyan hatóanyagot, amit a kutatási partnerünk irányításával klinikai körülmények között is teszteltek (CARVIN vizsgálat) és ígéretes eredményekkel zártak. Végül, de nem utolsósorban, több vegyület vált elérhetővé kutatócsoportunk számára, ami további tudományos diákköri munkák, doktori értekezések alapjául is szolgálhatnak.

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9. List of publications

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