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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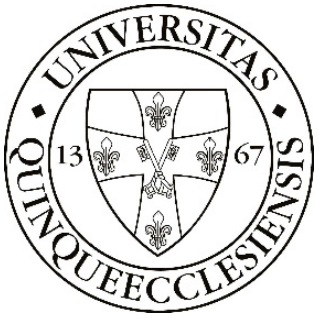

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1. INTRODUCTION

In December 2019, there was a high incidence of hospitalization 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). Within a short time, SARS-CoV-2 spread all around the globe infecting hundreds and thousands of people. On the 11th of March in 2020 WHO declared the Coronavirus disease-19 (COVID-19) outbreak to be a pandemic. The number of reported positive cases grew minute by minute. Lots of patients were transferred to the intensive care unit. Fatalities were taking on alarming proportions [1,2].

SARS-CoV-2 has an approximately 30 kb, positive-sense, single-stranded, nonsegmented RNA genome (+ssRNA) that encodes 16 non-structural (nsps), several accessory and 4 structural proteins (spike (S), membrane (M), envelope (E) and nucleocapsid (N)). The accessory proteins can modulate host cell metabolism and antiviral immunity. Nsps have key roles in viral replication by forming replication and transcription complexes (RTC), triggering double-membrane vesicle formations, generating GpppA cap on the 5'end of the viral genome, and proofreading the transcribed RNA. Nsps are also important in modulating innate immune responses. Structural proteins wrap the viral genome. During replication E and M proteins help the assembly of new viruses. N protein modulates the unwinding of the viral genome after entry. 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). 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.1). Conformational changes are followed by the fusion of the viral and host membranes (Fig.1). The released viral RNA is then first translated into two polyproteins, pp1a and pp1ab. These two polyproteins are then processed into several non-structural proteins by nsp3 and nsp5 (Fig. 1). The released nsps then form the RTC and facilitate immune evasion. Nsps are also form different membrane structures (double-membrane vesicle, convoluted membranes, double-membrane spherule) to create a protective microenvironment for viral genome replication and transcription. The newly generated genomic viral RNAs interact with the N protein. Then, together with the S, E, and M proteins, they form new viruses in the endoplasmic reticulum Golgi intermediate compartment. Ultimately, the newly assembled, matured viruses egress from the host cells through exocytosis and infect other

susceptible cells (Fig.1). 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 (Fig. 1). 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 [2–4].

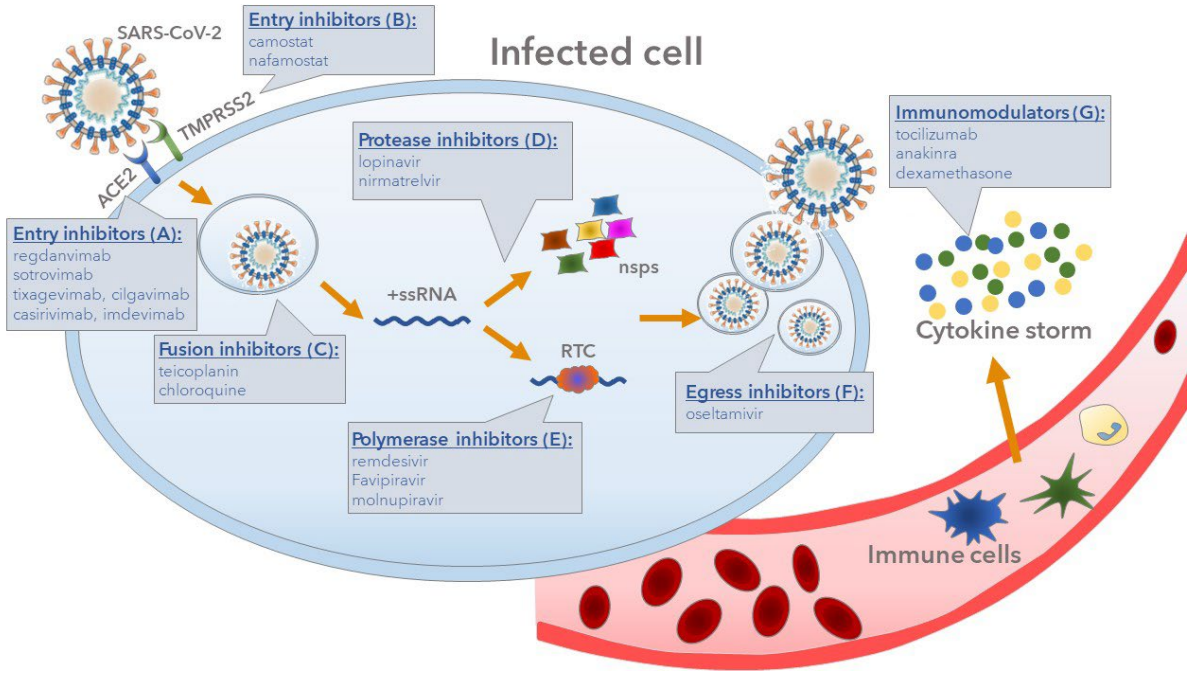

Figure 1. Targets of authorised COVID-19 therapeutics and a few proposed antivirals

Antivirals against SARS-CoV-2 target mainly non-structural proteins such as the RNA-dependent RNA polymerase (RdRp), main protease (Mpro), and papain-like protease (PLpro), and the interaction between spike and host proteins such as ACE2, TMPRSS2 and cathepsins (Fig.1). 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) (Fig.1) [5]. 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 SARS-CoV-2 variants can lead to immunisation 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. AIMS OF THE STUDY

The main objective of the work was to find compounds that effectively inhibit the newly emerged coronavirus, the SARS-CoV-2 *in vitro*. We 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. Before starting the antiviral research, we needed to successfully propagate the SARS-CoV-2 and determine the titer of the generated viral stocks. Furthermore, a reliable antiviral testing system also needed to be established.

After determining the reliability of our antiviral testing settings we:

1. Focused on testing compounds that were predicted to be effective, based on either *in silico* analyses or literature data.
2. Prioritized drugs that already had well-described pharmacokinetic profiles to mobilise them for the treatment of COVID-19.
3. Validated the reliability of a novel computational approach that can ease the search for functional building blocks.
4. Tested different newly synthesised derivatives of glycopeptide antibiotics to find the most suitable modifications for antiviral activity.

3. MATERIALS AND METHODS

3.1. VIRUS AND CELL LINES

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. Mycoplasma-free mammalian, adherent cells were used in our studies: the Vero E6 (ECACC, UK) and the A549 Dual™ hACE2-TMPRSS2 cells (InvivoGen, USA) which overexpress human ACE2 receptor and TMPRSS2. The cells were maintained in 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). Besides HI FBS different antimicrobial agents were added to the cell culture to avoid bacterial or fungal infections (1 % (v/v) penicillin-streptomycin (PS) (Lonza Group Ltd, Switzerland)) and in the case of the genetically modified A549 cells these were essential for the overexpression of hACE2 and TMPRSS2 (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 % CO_2.

3.2. Determination of Antiviral efficacy

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). After 30 min incubation at 37 °C in a 5 % CO_2 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). Forty-eight hours post-infection virus-induced cytopathic effect was examined under an inverted microscope (Eclipse Ti2-U, Nikon, Japan). Then total RNA was extracted from the supernatant of the samples. RdRp gene copy number was determined from the samples utilising the QX200 Droplet-Digital PCR system (Bio-Rad Laboratories, USA). The obtained data were used to generate IC_{50} curves that represent the concentration at which the compound exerts 50 % inhibition. GraphPad Prism 8 software (GraphPad Software, USA) was used to determine IC_{50}.

4. New scientific results

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.

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

Rucaparib (Rubraca®) is an FDA/EMA-authorised poly-(ADP-ribose) polymerase (PARP) inhibitor; currently used in antitumor therapies [6]. We proved that it can hinder the replication of SARS-CoV-2 in Vero E6 cells. To the best of our knowledge, we reported first this observation. We found that below 40 μM rucaparib does not toxic; its CC_{50} was 64.8 μM. After determining the non-toxic concentrations of rucaparib, the anti-SARS-CoV-2 effect was examined. The IC_{50} 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[7]. We presume that direct-antiviral activity would not be possible by the currently applied dosing regimen and administration route. To further study the *in vitro* antiviral activity of rucaparib, we also tested its activity in A549 Dual™ hACE2-TMPRSS2 cells. Cell viability tests revealed that 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. More studies would be beneficial to conscientiously recommend rucaparib as a direct-acting antiviral against SARS-CoV-2. More cell culture models should be applied, moreover, aerosol formulation or combinational studies should also be conducted.

4.2. AZELASTINE HYDROCHLORIDE BLOCKS SARS-COV-2 REPLICATION *IN VITRO*

A pathway-based computational approach led to the discovery of the potential polypharmacological properties of azelastine HCl. 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). Cell viability measurements revealed that 25 μM azelastine HCl did not cause damage in Vero E6 metabolism and morphological changes were also not detected. We used two different settings to measure the azelastine HCl activity against SARS-CoV-2; co-administration and therapeutic. Co-administration of the virus and azelastine HCl resulted in an IC_{50} 2.2 μM. Antiviral activity was also determined in the case when the cells were treated only after the initial infection (therapeutic setting). Azelastine HCl could exert antiviral activity, although it was a bit less effective; the IC50 value was 6.5 μM. These SARS-CoV-2 inhibitory concentrations were lower than those in the commercially available azelastine HCl-containing medications. 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 phase II clinical trial (CARVIN) [8].

4.3. METHYLENE BLUE INHIBITS SARS-COV-2 REPLICATION *IN VITRO*

Methylene blue is used for treating methemoglobinemia, preventing urinary tract infections in elderly patients and for intraoperative visualisations. Moreover, methylene blue and light-activated methylene blue can inactivate, for instance, hepatitis C virus, West Nile virus, human immunodeficiency virus 1, herpes simplex, human parvovirus B19, and simian vacuolating virus 40. Thus, it has been used to treat blood products before transfusion. 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 [9]. We aimed to confirm the observed inhibitory potential using infectious SARS-CoV-2. We found that methylene blue effectively blocks (IC_{50} of 1.70 μM) the replication of SARS-CoV-2 (B.1.5) in Vero E6 cells. 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.

4.4. FRAGMENT-BASED DRUG DESIGN SUCCESSFULLY IDENTIFIED SARS-COV-2 INHIBITING FRAGMENTS

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 be found on mammalian cells and pathogens. 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. 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. The identified small molecules were tested against SARS-CoV-2 *in vitro*. 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. EC_{50} value of the 3CLpro binding, aryl-piperazine fragment was 304 μM. Amongst the hits of nsp3 macrodomain inhibitors, two proved to be effective with EC_{50} 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 more compounds towards the distal ribose site.

Further optimisation of those fragments would enable the generation of highly specific antivirals.

4.5. GLYCOPEPTIDE ANTIBIOTIC DERIVATIVES CAN BLOCK THE *IN VITRO* REPLICATION OF SARS-COV-2

Glycopeptide antibiotics such as teicoplanin, dalbavancin, oritavancin, vancomycin, and telavancin are used to treat Gram-positive bacterial infections. Several studies showed that glycopeptide antibiotics and their derivatives have antiviral activities against a wide range of viruses [10]. We also tested different glycopeptide antibiotic derivatives, synthesised by Prof. Anikó Borbás's group. Teicoplanin pseudo aglycone, ristocetin aglycone and teicoplanin conjugated with apocaroteoids 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 (IC_{50} 1.8 μM). 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*. IC_{50} of bixin and β-apo-8'-carotenoic acid were 5.9 and 15 μM, respectively. 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. More studies involving various derivatives of glycopeptide antibiotics would be advantageous to develop good clinical candidates for antimicrobial therapies.

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6. PUBLICATIONS

6.1. PUBLICATIONS RELATED TO THE THESIS TOPIC

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6.2. ORAL AND POSTER PRESENTATION RELATED TO THE THESIS TOPIC

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