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Study of antiviral activity of diazadispiroalkane compounds against *alpha-* and *beta- coronaviruses*

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This thesis is based on several manuscripts that were published during my PhD.

The present PhD thesis has been completed during my enrolment as aPhD student at the Department of Biomedical Sciences, University of Sassari, Italy, from 1stNovember 2017 to 31stDecember 2021, under the supervision of Professor Pier Luigi Fiori.

The studies described in this thesis were conducted at the Department of Biomedical Sciences, University of Cagliari, under the supervision of Professor Aldo Manzin and Dr. Giuseppina Sanna.

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Dessì, D., Margarita, V., Cocco, A. R., **Marongiu, A.**, Fiori, P. L., &Rappelli, P. (2019). Trichomonas vaginalis and Mycoplasma hominis: new tales of two old friends. *Parasitology,* 146(9), 1150–1155. https://doi.org/10.1017/S0031182018002135

Margarita, V.*, **Marongiu, A.***, Diaz, N., Dessì, D., Fiori, P. L., & Rappelli, P. (2019). Prevalence of double-stranded RNA virus in Trichomonas vaginalis isolated in Italy and association with the symbiont Mycoplasma hominis. *Parasitology* re, 118(12), 3565–3570. https://doi.org/10.1007/s00436-019-06469-6

Madeddu, S.*, **Marongiu, A.***, Sanna, G., Zannella, C., Falconieri, D., Porcedda, S., Manzin, A., & Piras, A. (2021). Bovine Viral Diarrhea Virus (BVDV): A Preliminary Study on Antiviral

Properties of Some Aromatic and Medicinal Plants. *Pathogens* (Basel, Switzerland), 10(4), 403. https://doi.org/10.3390/pathogens10040403

* equal contribution

Madeddu, S.; Ibba, R.; Sanna, G.; Piras, S.; Riu, F.; **Marongiu, A.;** Ambrosino, A.; Caria, P.; Onnis, V.; Franci, G.; Manzin, A.; Carta, A. Human Enterovirus B: Selective Inhibition by Quinoxaline Derivatives and Bioinformatic RNA-Motif Identification as New Targets. Pharmaceuticals 2022, 15, 181. https://doi.org/10.3390/ph15020181

Participation in meetings and conferences

- 46° Congresso Nazionale della Società Italiana di Microbiologia 2018 – Palermo27-28/09/2018 (**Two posters**: "Metronidazole resistance in *Trichomonas vaginalis*: the role of the symbiont *M. hominis*" and "*Candidatus Mycoplasma girerdii*: a new friend for *Trichomonas vaginalis* into vaginal microbiome communities")

- The 5th International PhD search Student Science Symposium Hue – Vietnam, 27th April 2018 (**Poster** "*Trichomonas vaginalis* and its symbionts: different points of view of the parasitic-host relationship."

- 5th NationalCongress of the Italian Society for Virology– ONE VIROLOGY ONE HEALTH – online 5-6/07/2021(**Poster:** "NEUTRALIZING ANTIBODIES RESPONSES AGAINST SARS-COV-2 IN SARDINIAN COHORT GROUP")

- 49° Congresso Nazionale della Società Italiana di Microbiologia 2021 –Virtual SIM online 16-17/09/2021 e 20-21/09/2021 (**Poster and oral presentation**"Rare biscoumarin with significant anti-HIV-1 activity from Hypericum roeperianum")

List of abbreviations

ACE2	Angiotensin-converting enzyme 2
ATCC	American Type Culture Collection
BSL	Biosafety laboratory
CC_{50}	50% cytotoxic concentration
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic acid
COVID	Coronavirus infectious disease
CPE	Cytopathic effect
D-MEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
E	Envelope
EC_{50}	Half maximal effective concentration
ERGIC	Endoplasmic reticulum - Golgi intermediate compartment
FBS	Fetal bovine serum
GAGS	Glycosaminoglycans
hAPN	human aminopeptidase N

HCMV	Human cytomegalovirus
HCoVs	Human coronaviruses
HE	Hemagglutinin-esterase
HIV	Human immunodeficiency virus
HS	Heparan sulfate
HSPGs	Heparan sulfate proteoglycans
M	Membrane
MEM-E	Minimum Essential Medium with Earle's salts
MERS	Middle east respiratory syndrome coronavirus
MOI	Multiplicity of infection
N	Nucleocapsid
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pp	Polyproteins
qRT-PCR	Quantitative Reverse transcriptase- polymerase chain reaction
RNA	Ribonucleic acid
S	Spike

SAR	Structure–activity relationship
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SI	Selectivity index
ss	Single stranded
$TCID_{50}$	50% tissue culture infective dose
TMPRSS2	Transmembrane protease serine 2

ABSTRACT

RNA viruses cause many human and animal infections characterized by worldwide spread, high mortality, and significant health costs. In the case of some RNA virus diseases, such as Polio, Measles, Yellow Fever, and others, there is the possibility of accessing effective immunoprophylaxis measures. Still, the incidence and severity of some viral diseases are to develop effective and selective therapies. This scenario is essential to develop and manufacture new and effective antiviral agents.Usually, existing antiviral agents target specific viral proteins, with a higher probability of developing drug-resistant, especially those with an RNA genome. The recent SARS-CoV-2 pandemic has moved the scientific world to pay particular attention to the *Coronaviridae* family, ssRNA (+) viruses that cause respiratory diseases.We focused our studies on Alphacoronavirus HCoV-229E and Betacoronavirus HCoV-OC43 andSARS-CoV-2.

These viruses use different cell receptors to enter the host cell. Recent studies have highlighted an additional mechanism of endocytosis upstream of the binding with the receptor, which involves the heparan sulfate (HS) present on the cell surface through an electrostatic interaction in which negative HS charges interact with the virus envelope, concentrating the virions and allowing the recognition of the secondary receptor.Therefore, this phase of the entry process is strategically interesting as it could be the target of broad-spectrum antivirals. Among the strategies used to block the HS-virus interaction, one particularly effective is the use of small cationic molecules that bind competitively to HS. Among these cationic molecules of particular interest are the

dispirotripiperazines and their diazadispiroalkane derivatives synthesized and kindly provided us by Prof. Vadim Makarov (Russian Academy of Sciences) and colleagues.

During my Ph.D. course, the main goal was to identify new antiviral compounds with broad-spectrum activity and study their mechanism of action. These objectives have been achieved through a multidisciplinary approach. We evaluated the toxicity and the antiviral activity of some diazadispiroalkanes, against HCoV-229E, -OC43, and SARS-CoV-2.

Subsequently, we then evaluated the selectivity and potency of these compounds, studied its mechanism of action, examined the kinetics of virus adsorption in the presence of the compound. After all, we verified whether our identified lead derivative could interfere with the penetration of the virus in the cells.

The lead compound was shown to be a SARS-COV-2 inhibitor and more robust against HCoV-OC43. The molecule acts during the pre-treatment and in the early stages of infection, causing a dose-dependent reduction in infectivity.

Therefore, the validated method for evaluating the antiviral activity of diazadispiroalkanes can be applied for the discovery and development of broad-spectrum antiviral drugs *in vitro*.

Introduction

Viruses cause many diseases and death worldwide.The increase in epidemics in recent years has greatly impacted human life, both from a loss of life and economic point of view.Due to climatic changes that determine the displacement of vectors or mutations in viruses (1), humans have often become the new host of viruses that initially only affected animals (2, 3). Over the past twenty years, we have seen many examples of epidemic outbreaks caused by arthropod-borne viruses: West Nile virus (4), Chikungunya virus (5,6, 7), Yellow Fever virus (8), Zika virus (9);viruses that have leaped species such as Ebola (10), subtypes H5N1 and H7N9 influenza A (11,12), swine H1N1 influenza (13,14),SARS coronavirus (15,16,17), MERS coronavirus (18,19) and the youngest, SARS coronavirus 2 (20, 21). In the current scenario of emerging and re-emerging viruses, the pulse for developing and producing new and effective antiviral agents is particularly demanding, especially after the recent pandemic caused by SARS-CoV-2.

Since the 1960s, with the discovery of idoxuridine (22), the first synthesized antiviral for topical use, a new landscape has opened towards the discovery of new antiviral agents capable of selectively inhibiting viruses without inducing toxicity.Based on the theory on which the action of idoxuridine was found, it took another 17 years for a highly selective antiviral chemotherapy against Herpes Simplex 1 and 2 to be established. In fact, in 1977, the synthesis of aciclovir [9- (2-hydroxyethoxymethyl) guanine], a nucleoside analog, ushered in a new era of antiviral therapy (23, 24). However, 90 drugs divided into 13 groups were approved (25) as specific and effective antiviral therapies to treat nine human diseases, with an emphasis on the urgency of discovering new treatments. If on the one hand, thanks to vaccines, we have the possibility of having specific strategies for a single pathogen,an immune response on the part of the host towards the microorganism; on the other, the existing antivirals may be active on several viruses, such as Ribavirin(26,27), Acyclovir (28) Remdesivir (29). Re-purposing known antiviral drugs against emerging viruses is a strategy underlying the search for broad-spectrum antivirals (30).Broad-spectrum antivirals may be divided into two groups: the first one acts against the host-cell machinery, essential for establishing an infection and viral replication; the second one, acts directly against a selected viral enzyme, inhibiting it with high potency and minimalinterface with host processes.

Since antiviral agents are small molecules that target a specific stage of the viral replication cycle, drug-resistant viruses are likely to appear, especially those with an RNA genome, which have a high ability to adapt to the host's immune system or antiviral treatment due to the rapid rate of replication andwide variation of the pre-existing genome (31).

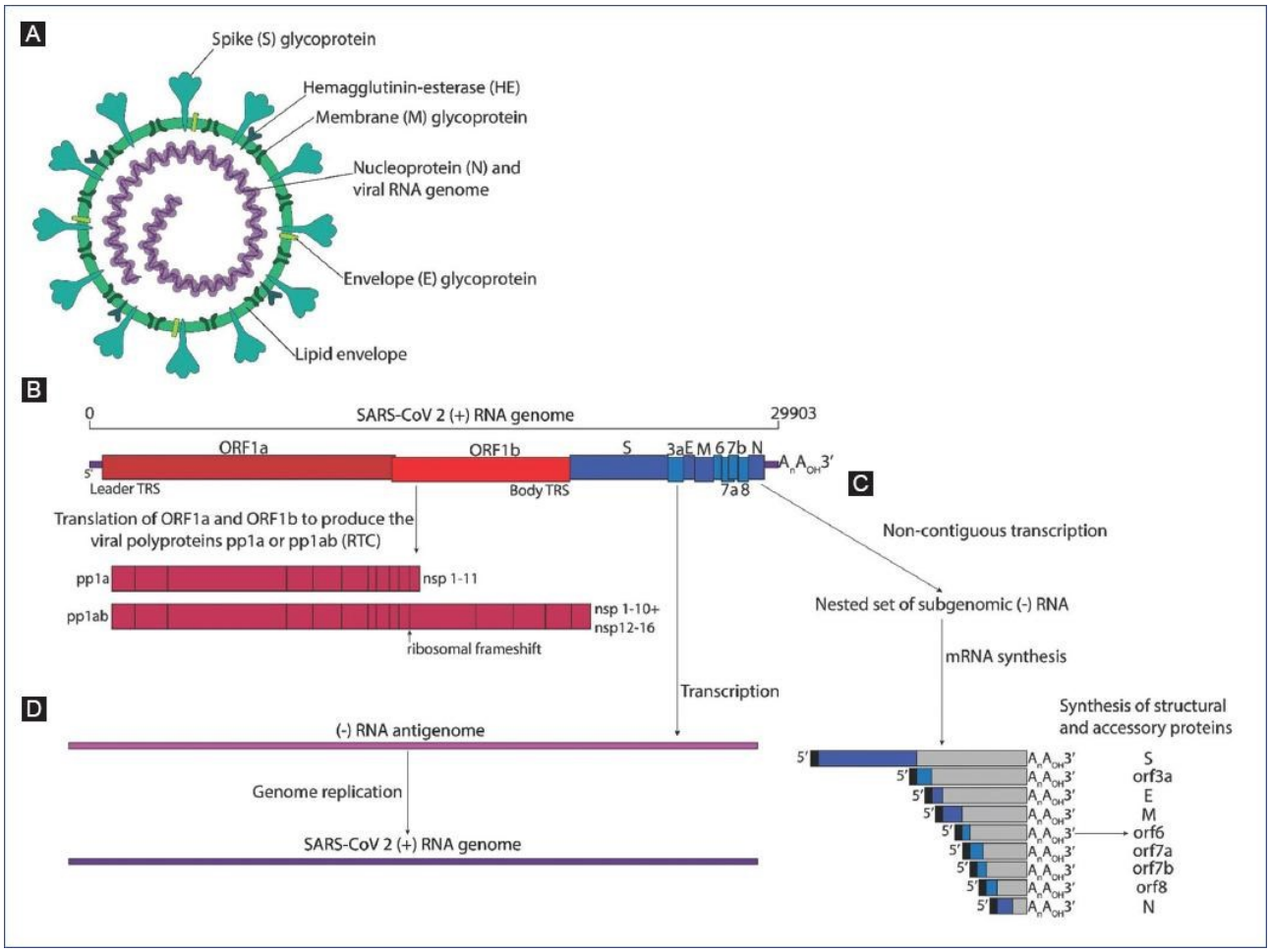


Figure 1: Coronavirus genome organization: structural (S – HE – N – M - E) and non-structural genes, polyprotein processing, RNA production and genome replication. Figure from Hidalgo et al., Bol Med Hosp Infant Mex. 2021 (32).

In this scenario, the urgency of discovering new pharmaceutical treatments emerges and the importance of knowing the underlying biology of viral infections. The pandemic events have forced the scientific world to pay specific attention to the *Coronaviridae* family, ssRNA (+) viruses that cause respiratory diseases. Human Coronaviruses (HCoVs) are members of the *Coronavirinae* subfamily, consisting of four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus*. Alpha- and betacoronaviruses originate from bats and infect mammals, while gamma- and deltacoronaviruses originate from birds and infect both bird and mammal species (33). Coronavirus genome (Figure 1) is the largest viral RNA described so far, with a size of about 27-32 kb that encodes for structural (S – HE – N – M - E) and non-structural proteins: spike (S), a type I transmembrane protein, which interacts directly with host cells through cleavage into an S1 subunit for receptor binding and an S2 subunit for membrane fusion from part of the host proteases.Heavy packed genomic RNA is wrapped forming a helically symmetricnucleocapsid (N). The nucleocapsid is covered by a phospholipid bilayer (envelope - E), composed of the S glycoprotein trimmer and the hemagglutinin-esterase (HE),which collaborates with protein S for the attachment of virions, thanks to its ability to bind sialic acid. The most abundant structural protein is membrane protein (M), which interacts with other viral proteins to assemble virions. The HCoV pp1a and pp1ab polyproteins (non-structural proteins) are processed autocatalytically by viral proteases into 16 non-structural proteins whose functions are to regulate and direct the replication and assembly processes of the virus (34).Replication cycle may be divided into several different steps. The entry, which we will discuss later, differs among the different coronaviruses. After the virus enters the

host cell,viral genomic RNA is released into the cytoplasm to allow translation of the two polyproteins, pp1a and 1 ab (35). The self-protein cleavage of polyproteins producesmore than a dozen nsps13, necessary to form a replicase - transcriptase complex to generate a new negative sense genomic RNA. The ssRNA(-) will became the templete for virions' genome. Meanwhile, the structural transmembrane proteins (S, M and E) are synthesized, inserted, and folded and folded and transported to the Endoplasmic reticulum-Golgi intermediate compartment (ERGIC), where the assembly of the virion will take place, once gRNA is encapsulated in the nucleoproteins produced. Once virions are formed, they are transported to the cell surface through the Golgi apparatus, via small vesicles (36, 37). The first coronaviruses to be described were CoVs 229E (*alphacoronavirus*) and OC43 (*betacoronavirus*) (Figure 2), causing mild upper respiratory tract infections, with the highest incidence during the winter season (38), though in fragile patients can lead to pneumonia (39).

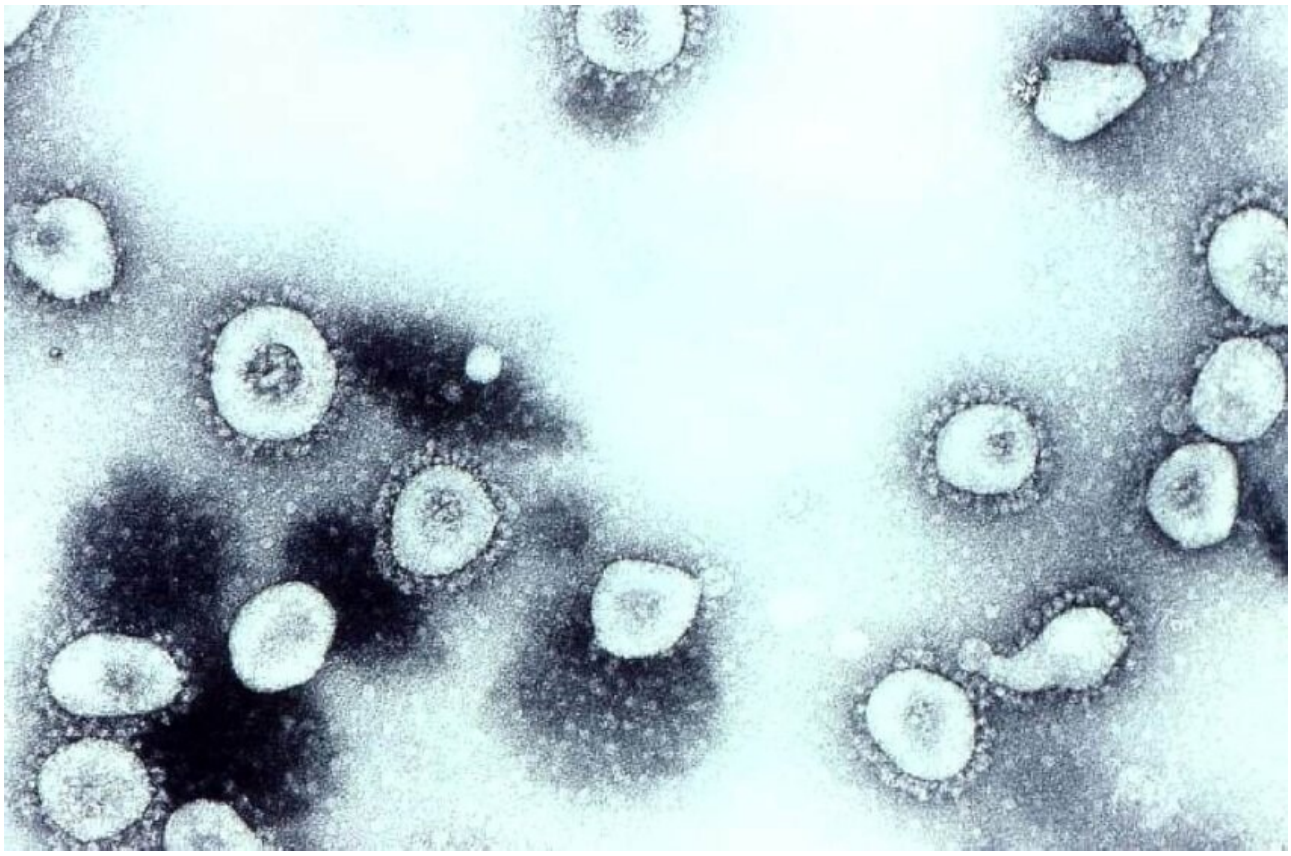


Figure 2: HCoV-OC43 observed usingtransmission electron microscopy (TEM).Photo Credit: CDC/ Dr. Erskine Palmer (1981)

After the emergence of SARS-CoV-1 in 2003, CoV-NL63 (40) and CoV-HKU1 (41) were identified, highlighting that these viruses were more clinically relevant than previously believed. With the discovery of MERS-CoV (42), we learn that spillover of species from animal to man could easily happen (43), thus becoming a major health risk. The confirmation of an increasingly frequent leap of emerging virus species from animals to humans came with the SARS-COV-2 pandemic, with a heavy toll onhuman lives.

Overall, known HCoVs studies have helped us understand its pathogenesis, replication cycle, and virus-host interactions to model strategies against the current pandemic.Our attention wasfirstfocused on two main representative viruses belonging to this family: HCoV-229E and HCoV-OC43. Then, we started studying SARS-CoV-2.These viruses share a marked tropism for the respiratory tract epithelial cells (44,45) and they can infect human neuronal tissue (46,47,48) (Figure 3).

Despite the various similarities these three viruses have in common, they differ strongly in the bond that the transmembrane spike glycoprotein (S) forms with host receptors and fuse viral and cell membranes to initiate infection.

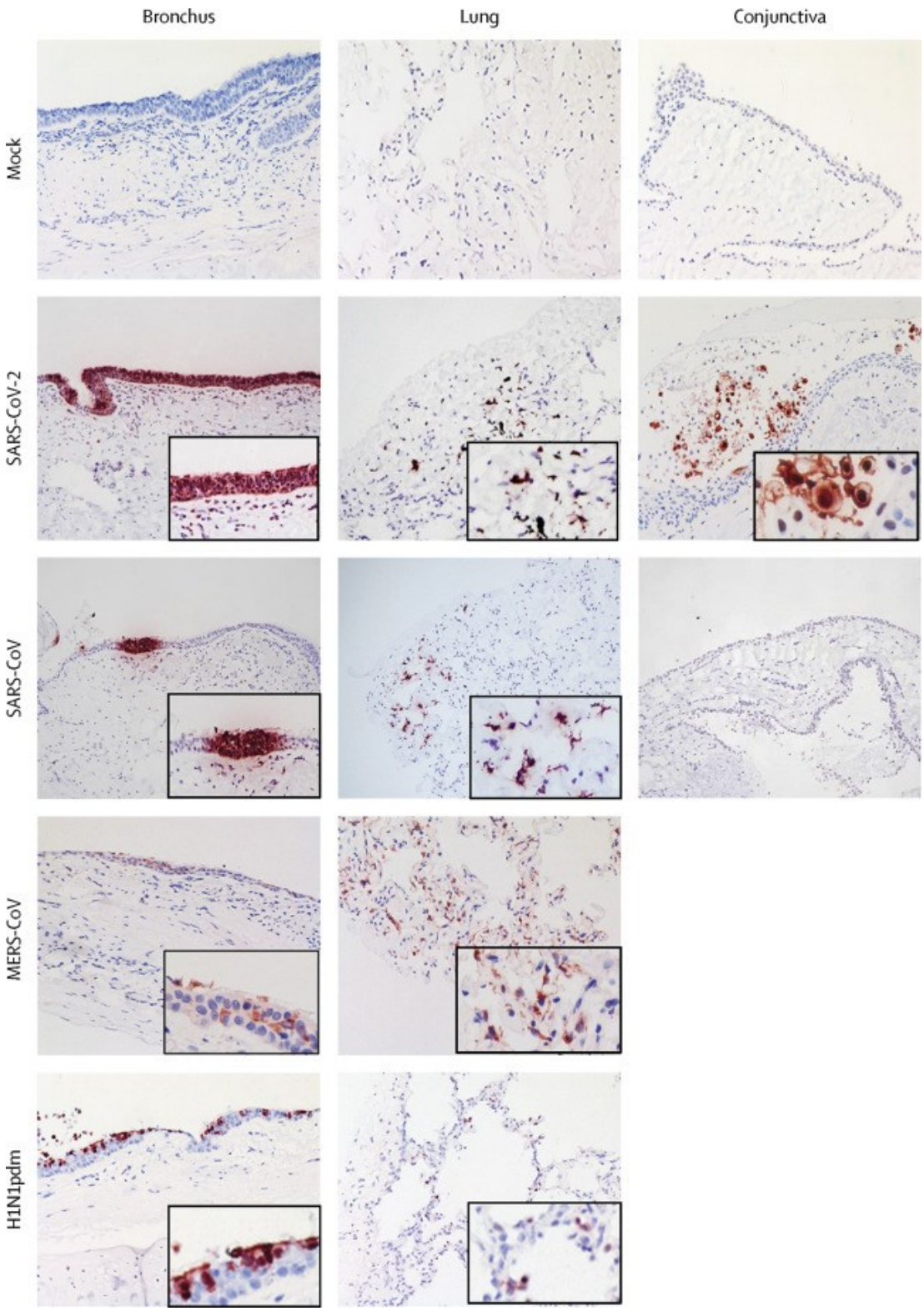


Figure 3. Tissue tropism of SARS-CoV-2, SARS-CoV and MERS-CoV. Figure from Hui KPY et al., Lancet Respir Med. 2020 (49)

HCoV-229 uses the cellular receptor human aminopeptidase N (hAPN),(50,51), a type II transmembrane protein and a zinc aminopeptidase also called cluster of differentiation 13 (CD13). HCoV-OC43 binds to sialic acids (9-O-Ac-Sias) attached as terminals to the glycan chains on glycoproteins and lipids(52), and the entry is via caveolin-1 dependent (53). SARS-CoV-2 uses the receptor of Angiotensin-converting enzyme 2 (ACE2) for entry and the serine protease TMPRSS2 for S protein priming (54). Recent studies suggest an additional mechanism of endocytosis upstream of these processes, involving heparan sulfate (HS) on the cell surface that facilitates receptor-mediated uptake of excess positive-bearing protein groups (55). Heparan sulfate proteoglycans (HSPGs) serve as an attachment factor for the initial anchoring of spike protein to the cell surface, which increases its local concentration to involve secondary receptors(Fig. 1) (56,57,58).So based on literature, one of the most attention-grabbing processes in discussing an effective antiviral is entry (59, 60, 61, 62). Viruses are obligate intracellular parasites; therefore, their replication results from the ability to enter the cell by endocytosis and through the host nucleus for replication and transmit the viral progeny from infected to non-infected cells. The viruses chosen, use different entry strategies into the cell as described above.

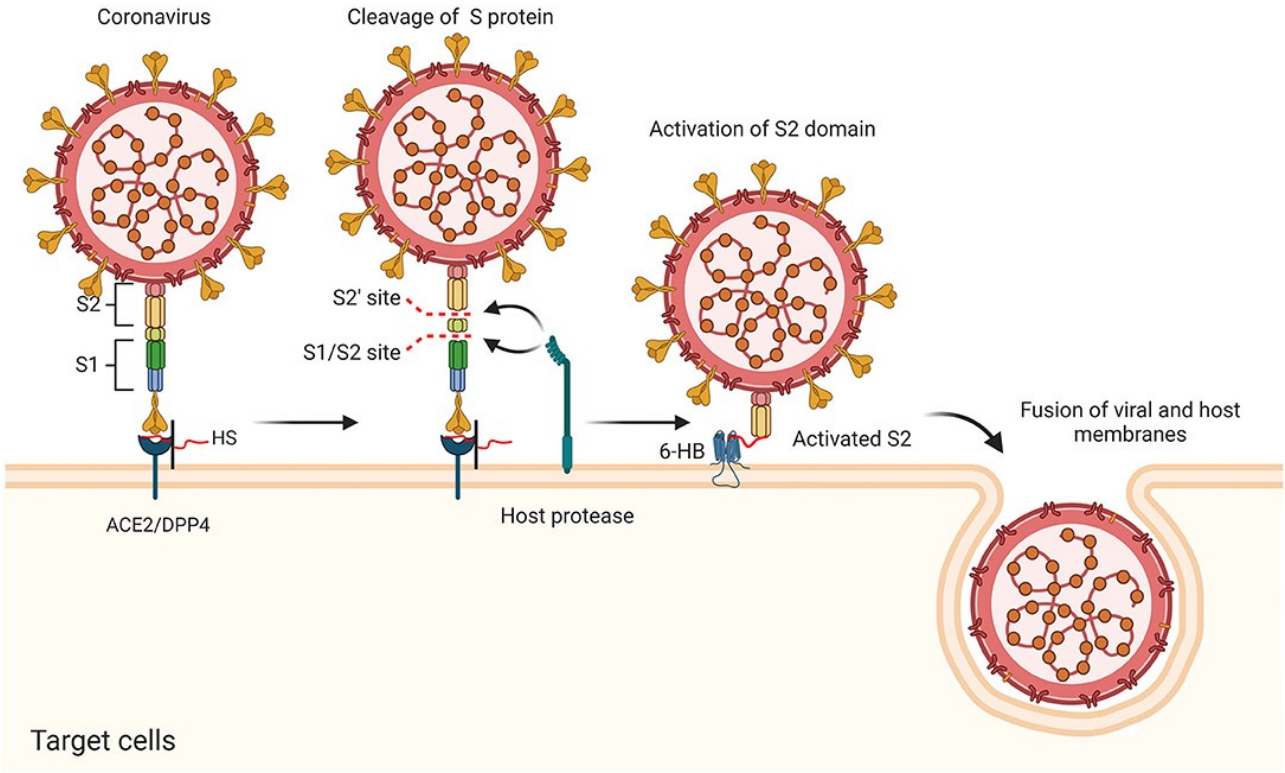


Fig. 4. Putative antiviral mechanism of CoVs during viral entry. Figure from Yu et al., *Front. Mol. Biosci.* 2021

Therefore, preventing the virus from joining the cell could be of great value. The binding between viruses and cells, in most cases, is mediated by surface proteins such as glycoproteins. Heparan sulfate (HS) is negative charge-enriched linear polysaccharide molecule that is attached to several membrane and extracellular proteins (HSPG), which are part of glycosaminoglycans (GAGs).

HS glycosaminoglycans (HSGAGs) are anionic molecules, due to the presence of sulfate and carboxylic groups, which interact withthe glycoproteins of the viral envelope. In fact, they behave as low affinity coreceptors that concentrate the pathogen on the cell surface and promote the binding with secondary receptors, which facilitate the entry of the virus into the cell (63) (Figure 4).

Over the years, several methods have been explored to block HSGAGs - protein interactions such as enzymatic alteration of HS, the use of competitive GAG mimetic compounds and small cationic

molecules that bind competitively to HS (64, 65).Among the latter, the most interesting are the dispirotripiperazine (DSTP27 and PDSTP) and their derivatives, (66)that showed an interesting antiviral activity against viruses belonging to different families(67).Briefly, dispirotripiperazines are part of the class of dispiro compounds, tricyclic molecules with two shared atoms, and are divided into piperazines (composed of three six-membered cycles), homopiperazines (composed of 2 six-membered cycles and a seven-membered cycle) or a dispiro system consisting of one six-membered cycle and two seven-membered cycles, synthesized by Soviet researchers, as they describe in the literature (68). They are mainly characterized by the presence of two quaternary positively charged nitrogen atoms in the structure, which are possibly responsible for electrostatic interactions with negatively charged HSGAGs, carrying out their antiviral activity (68, 69).

Based on a dispirotripiperazine-core, diazadispiroalkanes were subsequently synthesized, which proved to be active against viruses of the *Herpesviridae* family (70).

Aim of the study

The recent pandemic outbreak of SARS-CoV-2 spread rapidly, and it is still ongoing, thus antiviral therapies are urgently needed. There are currently no drugs for HCoV-OC43, which as mentioned, seasonally causes self-healing colds, which in fragile patients can lead to pneumonia (40).

For SARS-COV-2, however, only a few drugs have been approved as effective and safe against infection. In aid to Remdesivir, Molnupiravir which was born to treat influenza infections, has also shown antiviral activity against SARS-COV-2 and may be available to the public in the second half of 2022(71, 72).PF-07321332, developed by Pfizer, is currently in clinical development (73).

RNA viruses encode a small number of proteins, and they are dependent on host cell pathways to create viral progeny.The small molecules described above have great potential as a broad-spectrum antiviral therapy.

In vitro studies of antiviral activity are the basis of *in vivo* animal researchand clinical trials.

Through a multidisciplinary approach that encompassed several aspects of molecular, cellular biology and virology, thethesis' objectives can be summarized in the following points:

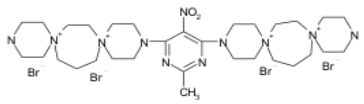
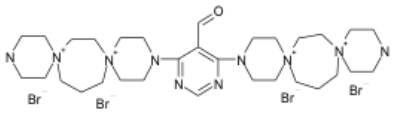
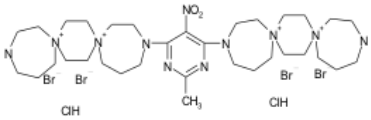
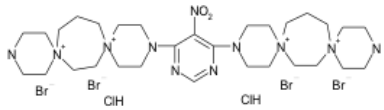
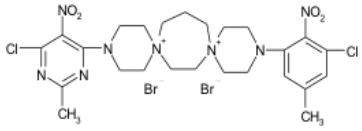
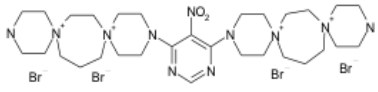
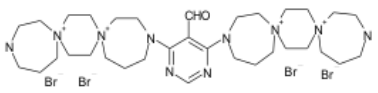
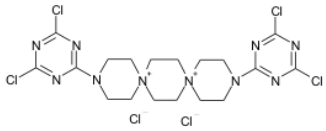
- Deepening the knowledge on *Coronaviridae* viruses HCoVs -OC43, -229E, SARS-CoV-2.
- To determine whether the diazadispiroalkane derivative compounds were cytotoxic for cell cultures that support viral replication.
- To evaluatethe potential antiviral activity of diazadispiroalkane derivatives on *alphacoronavirus* HCoV-229E, *betacoronavirus* HCoV-OC43 and SARS-CoV-2.

- To elucidate the molecular mechanism and potential broad-spectrum antiviral activity of the identified lead compound **236**.
- To provide more insight into the mechanism by which diazadispiroalkanes could inhibits HCoVs 229E, OC43 and SARS-COV-2 infection*in vitro*.

MATERIALS AND METHODS

Molecules

Diazadispiroalkane derivatives structures object of our study have been synthesized and kindly provided by Prof. Vadim Makarov (Russian Academy of Sciences) (Table 1).

Compounds	Molecular structure
11826228	
11826229	
11826235	
11826236	
11826237	
11926056	
11926061	
11926068	

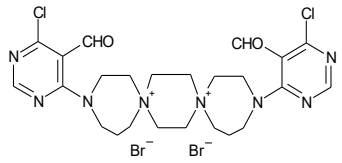
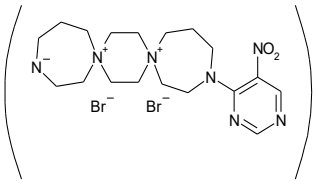
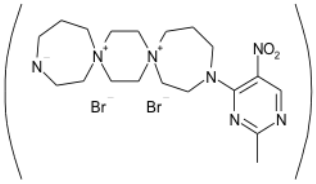
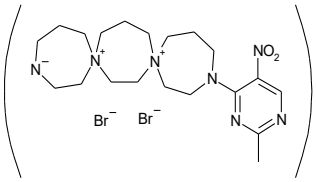
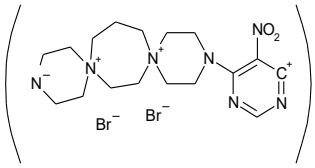
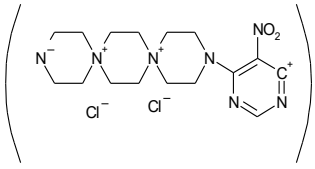
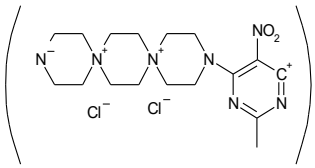
11926072	
12026126	
12026127	
12026128	
12026130	
12026131	
12026132	

Table 1. Structures of diazadispiroalkane derivatives.

Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of Coronaviruses were the following: Monkey kidney (Vero-76) [ATCC CRL 1587 Cercopithecus Aethiops], Monkey kidney (Vero C1008, clone E6) [ATCC CRL 1586 Cercopithecus Aethiops].

Human coronaviruses, single-stranded RNAs (ssRNA+) were *Coronaviridae*: *Betacoronavirus* strain OC43 (ATCC VR- 1558), *Alphacoronavirus* strain 229E (ATCC VR- 740); *Betacoronavirus* SARS-CoV-2 (strain VR PV10734) clinical isolate, kindly provided by Lazzaro Spallanzani Hospital, Rome, Italy.

All experimental work involving SARS-CoV-2 virus was performed in a biosafety level 3 (BSL3) containment laboratory.

Cytotoxicity assays

Vero-E6 cells were seeded in 96-well plates at an initial density of $3x10^5$/ml, in Minimum Essential Medium with Earle's salts (MEM-E), L-glutamine, 1mM sodium pyruvate and 25 mg/L kanamycin, supplemented with 10% fetal bovine serum (FBS). Vero-76 cells were seeded in 96-well plates at an initial density of 3x 10^5 cells/mL, in Dulbecco's modified Eagle medium (D-MEM) Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA. with l-glutamine and 25 mg/L kanamycin, supplemented with 10% FBS. Cell cultures were then incubated at 37 °C in a humidified, 5% CO2

atmosphere, in the absence or presence of serial dilutions of test compounds. The test medium used for the cytotoxic assay as well as for antiviral assay contained 1% of the appropriate serum.

Cell viability was determined after 72 or 144 hrs at 37 °C by MTT method (74).

Antiviral assays

Compound's activity against HCoVs229E and OC43 was based on inhibition of virus-induced cytopathogenicity in Vero-76 cells respectively, acutely infected with a MOI of 0.01. After a 3 or 5-days incubation at 35 °C, cell viability was determined by the MTT method, as described previously (74) Compound's activity against and SARS-CoV-2 was determined by plaque reduction assays in infected cell monolayers, as described (75).

Briefly, the monolayer of Vero-76 cells was grown overnight on a 24-well plate. The cells were then incubated with 100 µL of proper virus dilutions to give 50–100 PFU/well, immediately followed by the addition of various concentrations of the samples. The medium was also added to non-treated wells as non-infected controls. After 1 h, the inoculum was removed, and infected cells were overlaid with 1.2% methylcellulose medium containing various concentrations of test samples and incubated for 72 hours at 37 °C. After 3 days, the overlay medium was removed, and the cell monolayer was fixed with 4% paraformaldehyde solution, permeabilized, and immunostained with crystal violet 1% (w/v)for plaque detection. Plaques in the controland experimental wells were counted. All the experiments were conducted in triplicate and average values were plotted.

Yield reduction assay

Vero-76 cells were inoculated with HCoV-OC43 at a MOI of 0.1 in maintenance medium and tested compounds at not cytotoxic concentrations. Following 2 hours adsorption period at 35°C and 5% CO2, the inoculum was removed and replaced with fresh medium containing the same concentration of tested compounds. After 144 hours at 35 °C and 5% CO_2 each sample was harvested and diluted with serial passages, starting from 10-1 up to 10-8. The titer of the serial dilutions of the virus-containing supernatant was determined by Reed and Muench method. Remdesivir and Hydroxychloroquine were used as reference compounds.

HCoV-OC43 and SARS-CoV-2 Virucidal activity assay

Title compound (20 μM) was incubated with $1x10^5$ $TCID_{50}$/mL of OC43 at either 4 or 35 °C for 1hour. The mixture without test sample was used as the control. At the end of the incubation period, samples were serially diluted in media and titers were determined on Vero-76 and Vero E6 cells at high dilutions, at which the compound was not active. Virus titers were determined by the Reed and Muench method in Vero-76 cells.

Cell pretreatment assay

Vero-76 and Vero E6 cell monolayers in 24-well plates were incubated with 20 μM concentration of the 11826236 compound or references (100, 20, 4, 0.8 μM) for 2 hrs at 4 °C. After removal of the compounds and two gentle washes, cells were infected with HCoV-OC43 and SARS-CoV-2. After virus adsorption to cells, the inoculum was removed and the cells were then overlaid with medium, incubated for 4 days at 35°C and 37 °C, and then virus titers were determined by Reed and Muench method and plaque assay, respectively.

Time of addition assay

The confluent monolayers of Vero-76 and E6 cells in 96 and 24-well tissue culture plates were infected for 1 hrs at room temperature with HCoV-OC43 and SARS-CoV-2 dilutions to give final m.o.i. of 1. After adsorption, the monolayers were washed two times with MEM medium with L-glutamine, supplemented with 1% inactivated FBS, 1mM sodium pyruvate and 0.025 g/L kanamycin (Maintenance Medium) and incubated with the same medium at 5% CO2 and 37 °C (time zero). Vero-76 and E6 cells were treated with compound 11826236 (20 μM, approximately 10 times higher than the EC_{50}) or reference for 1 hours during infection period (at -1 to 0) and at specific time point, 0 to 2, 2 to 4, 4 to 6, 6 to 8, and 8 to 10 hrs post infection. After each incubation period, the monolayers were washed two times with maintenance medium and incubated with fresh medium until 10 hrs post-infection. Then, the monolayers were frozen at -80°C and the viral titers were determined by Reed and Muench (HCoV-OC43) and plaque assay (SARS-CoV-2).

Adsorption assays

Vero-76 cells and E6 grown in 96 and 24-well plate were infected with OC43 and SARS-CoV-2respectively, with a MOI of 0.1, in the presence or absence of compound 11826236. Multiwell were incubated for 60 min at 4°C. Medium containing unadsorbed virus was then removed, cells were washed twice with PBS and overlayed with medium. Plaques were counted after 72 hrs of incubation at 37 °C for SARS-CoV-2 while titer reduction was determined after 144 hrs of incubation at 35 °C by Reed and Muench method for HCoV-OC43.

Penetration Assay

A 24-well tissue culture plate was seeded with Vero-E6 cells (3 x 10^5 cells/well), which were then incubated overnight at 37 °C under 5% CO2. The cells were chilled on ice for 1 h, and the medium was removed. The cells were infected with SARS-CoV-2 on ice for 1 h. The medium containing unbound virus was then removed; various concentrations of 11826236 (100-0.8uM) in medium were added, and the cells were incubated at 37 °C for 1 h to trigger endocytosis of the virus.

The infected cells were then treated with alkaline phosphate-buffered saline (PBS; pH 11) for 1 min to inactivate any viruses that had not penetrated the cells, and then acidic PBS (pH 3) was immediately added to neutralize the mix. The neutralized medium was removed, cells were overlayed with 0.75% methylcellulose in media and then were incubated at 37 °C. After incubation at 37°C for 72 days cells were stained, and plaques determined by counting.

Quantitative reverse transcription PCR (qRT-PCR)

Total viral RNA was extracted 48 hours post infection in the presence or absence of compound 11826236 using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting cDNA was used as a template for PCR.For quantification, a standard curve was performed using 10-fold dilutions of viral stocks of HCoV-OC43 cDNA.qRT-PCR was performed using TaqMan™ Universal PCR Master Mix according to the manifacturer's protocol.

The PCR primers' set were designed on N gene:

	Forward	Reverse
HCoV- OC43	GTGCAGGAAGGTCTGCTCC	TCCTGCACTAGAGGCTCTGC

The cycle conditions of qPCR were 2minat50°C, 95°C 10 min, followed by 40 cycles of 95°C 30 sec and 60°C for 30 sec. A melting curve was performed by a gradual increase in temperature (0.5 °C). Real-time PCR assays were run on a CFX96 Touch^{TM} Real-Time PCR Detection System (Bio-Rad). The resultant curves were analyzed by Biorad CFX Manager 3.1. Each experiment was performed in triplicate.

Statistical analysis

Cell-based experiments were independently repeated at least three times. The data are reported as mean ± standard deviation (SD). Data were analyzed using the GraphPad Prism v5.0 software (La Jolla, CA, USA). Statistical analysis of the experimental results was performed using unpaired Student t-Test.The statistical significance values are defined as *p< 0.05, **p< 0.01, ***p< 0.001.

RESULTS Part I, Broad H-CoVs antiviral studies

Diazadispiroalkane derivatives were evaluated for their potential broad-spectrum anti-Coronaviruses activity in cell-based assays. Several compounds exhibited significant inhibitory activity against HCoV-OC43 and SARS-CoV-2, with EC_{50} values in the low micromolar range (i.e., <10 μM, Table 2).

No antiviral activity was detected against HCoV 229E. Concomitantly, moderate to low cytotoxicity was detected for almost all compounds, with CC_{50} values mostly in the high micromolar range (>100 μM) in Vero-76 cells. Concerning the spectrum of antiviral activity, the most relevant results referred to the interesting activity of derivatives 11826**228,** 11826**236**, 11926**056**, 12026**128** and 12026**131** against the panel of HCoV-OC43 and SARS-CoV-2, while as shown, none of the derivatives exert antiviral activity against HCoV 229E (Table 2).

Particularly, **236** resulted in being effective in reducing cytopathy induced by HCoV-OC43 with an EC_{50} of 2 μM and a selectivity index, SI >50.

Compounds	Vero-76 $^{a}CC_{50}$	SARS-CoV-2	OC43[c] $^{b}EC_{50}$	229E
11826228	>100	8.6	8.5±2	>100
11826229	>100	30	65	100
11826235	>100		7±2	>100
11826236	>100	**1.3±0.3**	**2±0.9**	>100
11826237	>100	85±5	>100	>100
11926056	>100	3	6±2	>100
11926061	>100	30	50	>100
11926068	>100	>100	>100	>100
11926072	>100	78.7±3	>100	>100
12026126	>100	>100	33	>100
12026127	>100	>100	13	>100
12026128	100	4.4±0.3	7±1.6	>100
12026130	39	7±0.9	7±0.9	>39
12026131	>100	3	13	>100
12026132	28	>28	6.8	>28
Remdesivir	>100	1.6±0.3	-	6±2
Hydroxychloroquine	40±5	1.2	9.8±17	-

Table 2. Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. [a]Compound concentration (μM) required to reduce the viability of mock-infected Vero-76 cells, as determined by the MTT method. [b]Compound concentration (μM) required to reduce the plaque number of SARS-CoV-2, 229E by 50% in Vero-76 monolayers and Compound concentration (μM) required to achieve 50% protection of Vero-76 cells from OC43 ([c]), induced cytopathogenicity, as determined by the MTT method.

The same remarkable antiviral activity was recorded for **236** against SARS-CoV-2 with an EC_{50} of 1.3±0.3 μM (SI > 77).

056 and **128** derivatives were able to reduce the plaque number of SARS-CoV-2, by 50% in Vero-76 monolayers with an EC_{50}ranging from 3 to 4.4 μM. Hydroxycholoquine was used as reference compound against HCoV-OC43 and under our experimental conditions showed an EC_{50} of 9.8 ±1.7 μM. Whereas, Remdesivir was used as reference compound against HCoV-229E and SARS-CoV-2 (EC_{50} of 6μM and 1.6 μM, respectively).

12026**130** retain the interesting antiviral activity againstHCoV-OC43 and SARS-CoV-2 but its cytotoxicity on Vero-76 monolayers resulted increased (CC_{50} = 39μM).

11826**229** and 11926**061** resulted ten times less active than the above-discussed derivatives both towards OC43 and SARS-CoV-2.

11926**126** and 11926**127**were able to maintain the anti-OC43 activity but were completely inactive on SARS-CoV-2.

11926**068** and 11926**072** instead, resulted inactive against all the strains tested.

Based on the described preliminary data, **236** showed the most compelling profile, resulting in a promising antiviral activity against SARS-CoV-2 and broaden spectrum againstHCoV-OC43.

Therefore, **236** was selected for additional studies, first against HCoV-OC43, and then against SARS-CoV-2, as reported below, to better understand its *in vitro* mechanism of action.

Lead 236 and HCoV-OC43 antiviral studies

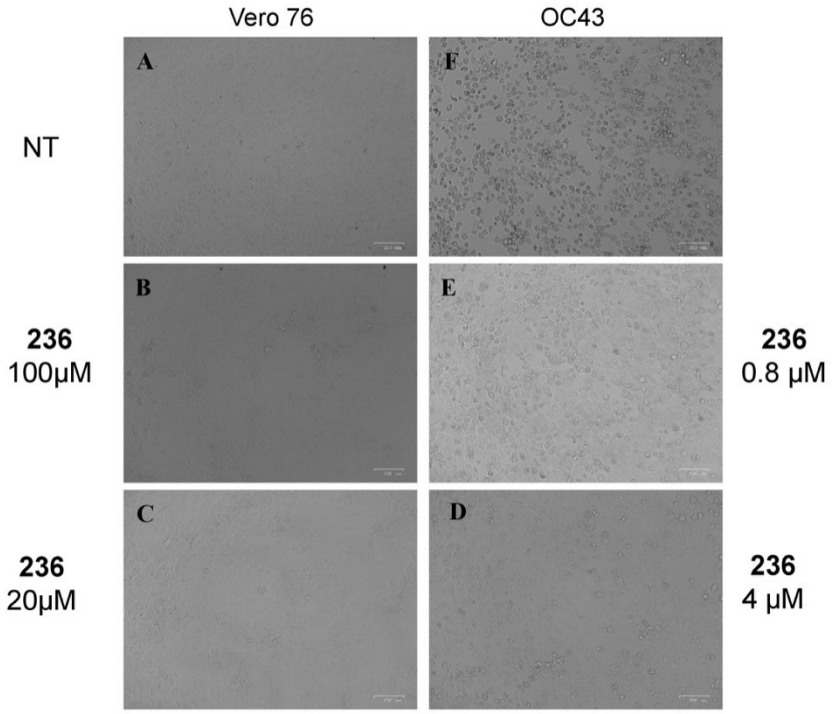


Figure 4. Effect of **236** inhibitor (100,20,4,0.8 µM) on the Vero-76-infected monolayers by HCoV-OC43. Control (A), infected treated cells with 100 µM (B), 20 µM (C), 4 µM (D), 0.8 µM (E) HCoV-OC43 infected cells (F). Pictures of cell morphology were taken at 5 days post-infection using ZOE Fluorescent cell imager (Bio-Rad) (bar size = 100 µm, magnification, 20 ×).

The protective effect of **236** on OC43 infected cells was shown in Panel figure 4. The intact cell monolayer suggests that the three higher concentrations determined protection from infection and no cytotoxic effect against cells (Fig. 4B, C and D), whereas, in 4D, OC43 infected monolayer showed a cytopathic effect with morphological variations and shape rounded cells representing the dead cells. The **236** CC_{50} and EC_{50} curves are reported on figure 5.

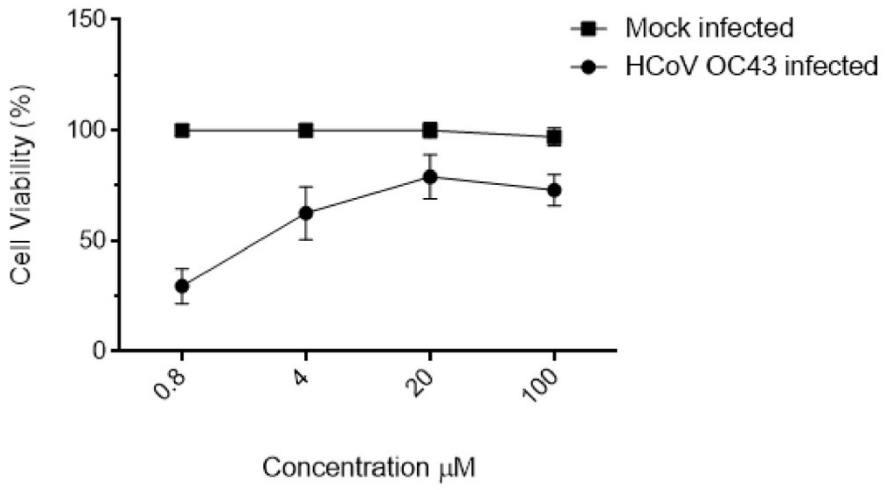


Figure 5. Cytotoxicity and anti HCoV-OC43 activity of 236 derivative. The viability of HCoV-OC43 infected Vero-76 cells was estimated by MTT assay, 6 days post-infection. The number of live cells was expressed as a percent of mock infected, non-treated, control cells. Data are expressed as means ± SD of at least three independent measurements.

To detect the reduction of virus titer in the presence of the **236**, lead derivative, a Yield reduction assay was conducted. Not cytotoxic concentrations of 100, 20, 4, and 0.8 μM were employed and was interesting observed a dose-dependent reduction of OC43 titer.

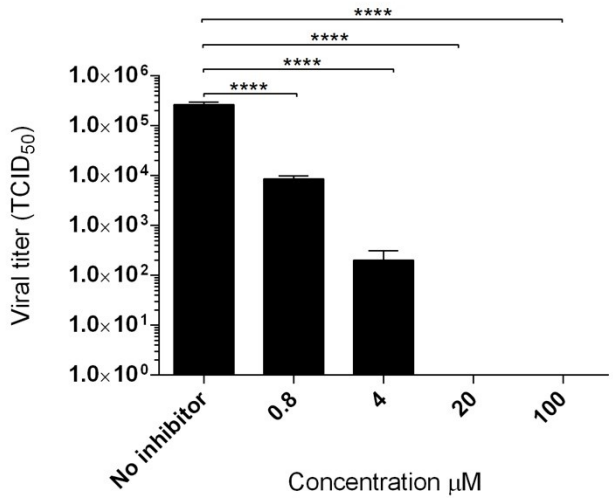


Figure 6. The Yield of infectious OC43 virus produced in infected Vero-76 cells treated with 236. Monolayers were infected with OC43 (m.o.i. = 0.1). The infected cultures were treated with 236 at indicated doses (100, 20, 4 0.8 μM). Viral yields in the culture supernatant were tittered at 48 h post-infection. Results are expressed as means ± standard deviations from 2 separate experiments. Statistical analyses were performed by Student t-test. Results were considered significant vs control when * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.

Anti-OC-43 activity of **236** was also investigated through Real-Time PCR protein (N) gene expression (Figure 7). In particular, **236** strongly reduced the N expression after OC43 infection until 4 μM, and it resumes being expressed in a dose-dependent manner at lower concentrations.

All together these results point out that 236 possesses a great antiviral and never investigated activity.

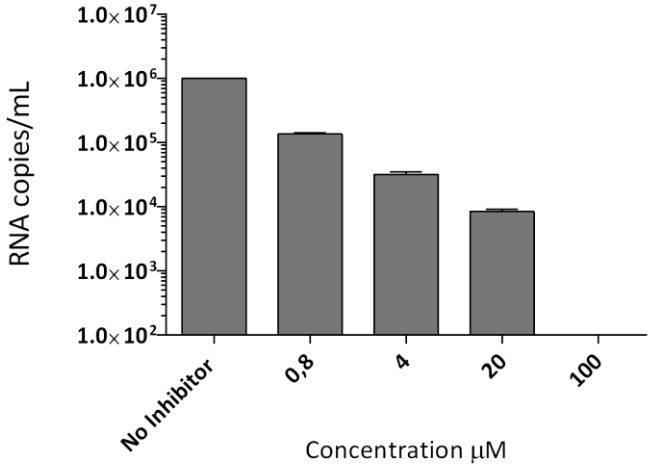


Figure 7. Real-Time PCR quantifying infection with HCoV-OC43 after 48h treatment with 236 derivative.

RESULTS Part I, SAR Studies and 236 potency against HCoV-OC43

The COVID-19 pandemic has shown the world how lack of therapies and detailed understanding of viruses limit effective countermeasures. We investigated the mechanism of antiviral action and the target pathways of small molecules, already effective against ssRNA (+) viruses (74).

Small molecules are opening a new scenario for broad spectrum antiviral therapies,especially to avoid drug resistance.

Starting from a structure–activity relationship (SAR) perspective, we can state the relevance of the core of compounds **236** and **056**, they have the same central structure, but one is a salt and the other is a base, for which similar antiviral activity is appropriate.

It is interesting that compound **228** with an additional methyl group in pyrimidine has slightly less activity, perhaps this methyl group creates difficulties for the electrostatic interaction of charged nitrogen with the sulfur groups of the proteoglycan sulfate.

Furthermore, the nitro group appears to play an important role in the activity of compounds and samples **229** and **061** in which nitro transformed into a fraction of the formyl group demonstrated lower activity on SARS-Cov-2 and HCoV-OC43.

Compounds **061** and **235** also have some changes in the structure of the nucleus, so here we built the diazadispiro system due to 7-6-7 rings which led to increased activity.

Interestingly, some polymeric compounds in which portions of diazadispiro alternate with pyrimidines also demonstrate antiviral activity, for example **131**, as described by Makarov and colleges against HSV-1 (66).

The derivative of interest (**236**) was firstly investigated against HCoV-OC43.

We did not observe morphological alterations of the cell monolayers of Vero-76, data confirmed by the MTT method. While, after infection, HCoV-OC43 showed a cytopathic effect with morphological variations of monolayer and round-shaped cells representing dead cells (Figure 4F). Conversely, treatment with various concentrations of the compound protected cells from HCoV-OC43 infection, up to 120 hpi (Figure 4B-E).

Subsequently, **236** was valued in a yield reduction assay, in order to establish the reduction of virus titre in the presence of the active compound and further demonstrate the powerful antiviral activity of this compound.

Not cytotoxic concentrations of 100, 20, 4, and 0.8 μM were used and a dose-dependent reduction of virus titre was observed (Figure 5).

Accordingly, the antiviral activity of **236** was also confirmed through a Real-Time PCR assay, in which we were able to quantify the reduction in viral RNA copies, 48 hours after infection, compared to the standard reference curve (Figure 7).

Indeed, the gene expression of the nucleocapsid (N) protein was strongly reduced after OC43 infection and treatment with 236 for up to 48h, and resumes being expressed in a dose-dependent manner at lower concentrations.

Protein N represents the nucleopcapside protein, encoded by the structural gene present in single copy in the OC43 genome (75). The quantification of RNA copies, performed by RT-PCR, is directly proportional to the number of viruses present in the treated and untreated sample.

The results obtained in Real-Time PCR confirm that compound 236 is able to neutralize the viral titer at low concentrations.

These preliminary data indicate that **236** possesses a great antiviral and never investigated activity, as that reported against HCoV-OC43. Since the compounds showed a different activity between *alpha* and *betacoronaviruses*, we wanted to evaluate whether SARS-CoV-2 showed the same profile of inhibition to diazadispiroalkans derivates.

RESULTS Part II, Lead 236 and SARS-CoV-2 antiviral studies and mode of action (MoA).

In *in vitro* assay **236** was endowed with an interesting activity against SARS-CoV-2 (1.3 μM) and a CC_{50} >100 μM on green monkey kidney epithelial cell (Vero-76).

Dispirotripiperazines, were found able to inhibit virus-to-host fusion by binding to the cell-surface heparan sulfates (64, 65, 68 76, 77).

Consequently, we hypothesized that the dispirotripiperazine-based molecules, **236** and other derivatives (Table1), might have the same molecular mechanism towards SARS-CoV-2, inhibiting viral adhesion to the cell.

To investigate this hypothesis, a virucidal activity assay was conducted, to evaluate the effect of compound **236** on SARS-CoV-2 viral infectivity. As expected, no direct influence of the compound was observed on virion infectivity, treated at two different temperatures. (Figure 8A).

Furthermore, we assessed the potential mode of antiviral activity with a time-of-addition assay (Figure 8B).

Remdesivir and Heparin were used as a reference compounds. The experiment was performed in SARS-CoV-2-infected Vero-76 cells (m.o.i. = 1) exposed to the compound (20 μM) at different times of infection.

Interestingly, Figure 8B showed that the compound exerted the greatest effect when added before infection and along with the virus. As expected, the antiviral activity of the selected compoundshowed activity comparable to heparin, used as a positive control.

Remdesivir, on the other hand, has been shown to be more active in later stages of the replication cycle, being a nucleoside analogue(29).

Furthermore, moderate titer reduction is detectable later, after the infection process (6 hpi), making us hypothesize that **236** compound can also interact in the cell-cell virus spread.

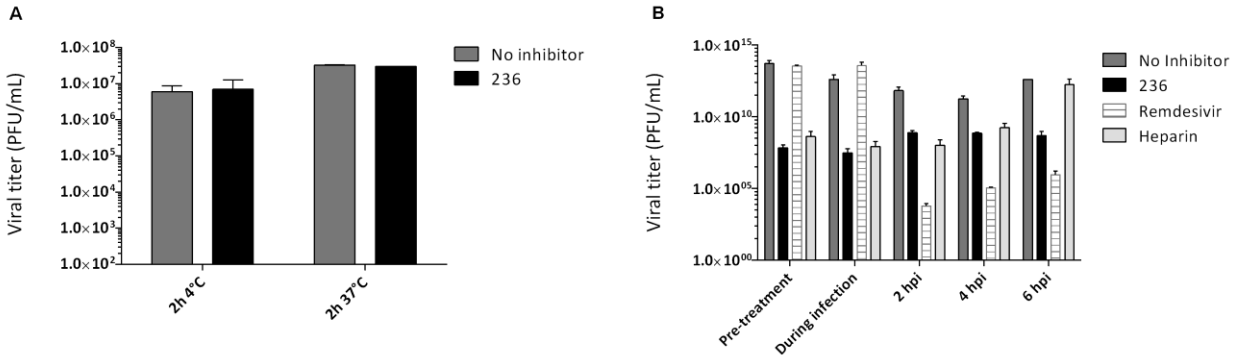


Fig. 8. Evaluation of mechanism of action (MoA) of 236 *in vitro.* (A) Virucidal effect (expressed as plaque-forming units (PFU/mL) of 236 (20 μM) against SARS-COV-2 at either 4 °C or 37 °C for 1 h. Dark columns, viral titer for viral and 236 solution; Grey columns for the viral titer of not treated solution. (B) Pre-Treatment Assay and Time course experiment. Vero cells were inoculated with SARS-COV-2 (m.o.i. = 1) and then 236 (20 μM) was added at the indicated times. Viral yields were determined by plaque assay. Dark columns, viral yield for cells treated with 236, Grey columns; the viral yield for control cells. Light grey and pattern color viral yield for cells treated with reference compounds, Heparin and Remdesivir, respectively.

The dynamic of SARS-CoV-2 adsorption in the presence of **236** was also evaluated.

The treatment at low temperature allowed the attach of viruses to the receptors present in the host cell surface, but avoided the following internalization of virions.

In view of that, Vero cell monolayers were incubated with SARS-CoV-2 (m.o.i. = 1) and compound **236** for 2 h at 4 °C, using the fixed and not cytotoxic concentrations employed in the antiviral assay (100, 20, 4, 0.8 μM).

The treatment with **236** determined a dose-dependent reduction of the virus infectivity.

Moderate reduction of adsorption was detected until the lowest concentration (0.8 μM) (Fig. 9A).

Dextran Sulphate was used as a positive control (78, 79).

Additionally, to investigate the mechanism of **236** inhibition of the SARS-COV-2 entry into Vero cells, we carried out a penetration assay.

Prechilled Vero cells were infected with SARS-CoV-2 (m.o.i. = 1) for 120 min at 4 °C.

Thereafter cells were overlayed with 100 μM, 20 μM of 236 or left untreated. Penetration was permitted at 37 °C for 60 minutes to activate endocytosis of the virus.

The infected cells were then treated with alkaline buffer (PBS; pH 11) for 60 seconds to inactivate any viruses that had not penetrated the cells, and then acidic PBS (pH 3) was instantaneously added to neutralize the mix. Dextran Sulphate was used as a control. The percentage of inhibition was subsequently determined (Fig. 9B).

In contrast to attachment, **236** reduced penetration process only up to 36 to 51 % at the higher concentration tested (Fig. 9B). Dextran Sulphate treatment led to a reduction of 36 % but at higher dose (100 μg/ml) (Fig. 9B).

These results suggest that **236** well prevents the attachment of SARS-CoV-2, whereas it had only some additive effect on penetration.

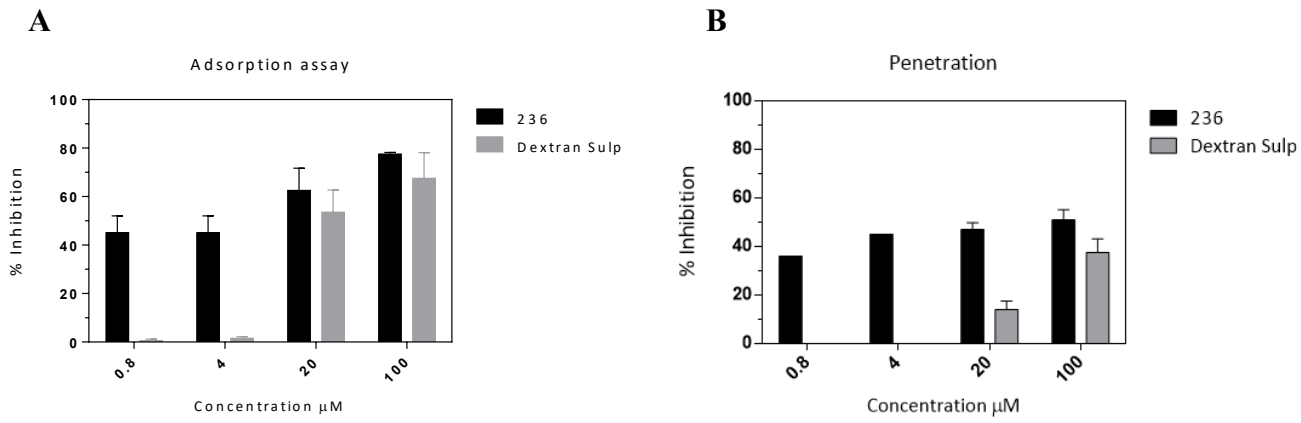


Figure 9. Adsorption (A) Assay Vero-76 monolayers were pre-adsorbed for 120 min at 4 °C with viruses at a m.o.i. = 1 in the presence of fixed concentrations of compound 236. **Penetration (B) assay**. Dose-dependent of 236 (Dark columns) inhibition of SARS-CoV-2 penetration. Dextran Sulphate, (Grey columns) was used as internal control. The results presented were obtained from three independent experiments. Data are mean ±SD.

DISCUSSION

Coronaviruses are classified in four genera. *Alphacoronaviruses, Betacoronaviruses, Gammacoronaviruses* and *Deltacoronaviruses*. Among them, only *Alpha* and *Betacoronaviruses* are pathogenic for humans (82).

Alphacoronaviruses and *betacoronaviruses* typically cause respiratory illness in humans and gastroenteritis in animals (83). Between alfa coronavirus, 229E is associated to upper and lower respiratory tract infections (84). while the beta HCoV-OC43 can be also neuroinvasive and may be associated with multiple sclerosis (85, 86).

They were not considered to be extremely pathogenic to humans until the occurrence of severe acute respiratory syndrome (SARS) in 2002. The introduction of new drugs for the prevention or therapy of Covid-19 in an already worned healthcare system is pivotal.

Especially in the context of persistently high morbidity and mortality due to COVID-19, efficacious antivirals have an evident appeal. Reduced viral load would decrease the severity and span of disease, as well as the possibility of transmitting the virus.

Among COVID-19 antivirals, resulted in advanced clinical trials (87), Molnupiravir made by MSD (Merck), that acts as a competitive nucleoside analogue for viral RNA dependent RNA polymerase, while PF-07321332, Pfizer's SARS-CoV-2 protease inhibitor co-packaged with ritonavir was authorized for emergency use for the treatment of high-risk patients with COVID-19 to prevent severe illness (88, 89).

Viruses that have short replication times and produce high numbers of copies, determining mutations can give resistance against antiviral drugs (25). SARS-CoV-2 shares this ability to produce frequent escape mutations, then multiple antiviral drugs may be required in combination for effective treatment or prophylaxis.

Therefore, an inhibitor targeting an early process of infection would give an alternative to available antivirals. One distinctive mode of action is the prevention of virus adsorption by defined blockage of heparan sulfate (HS) receptors (57). Drugs from different classes can thus be combined to generate a synergized activity against SARS-CoV-2-induced cytopathic effect (90).

HSPGs are capable to support many viruses in invading the host cells during different steps of their life cycle. Due to their ubiquitous expression on the cell surface, tissue and functions, viruses utilize HSPGs primarily for the attachment, the internalization, trafficking, and spread.

Among the viruses, the contact between the host cell surface and HSPGs is a precondition for penetrating and infecting target cells. Different types of coronaviruses, including human coronavirus OC43, and severe acute respiratory syndrome coronavirus-2, require before the specific interaction with the entry receptors, a preliminary binding of the viral envelope proteins with host cell carbohydrates or glycoproteins, this allows the increase in the number of viral particles on the cell surface and then guides their internalization.

Other studies (91) previously showed that HCoV OC43, resulted able to bind, not only the O-acetylated sialic acid, but also HS in cell culture. This affinity for HS has been described from Chamberlain K., and coworkers as a resultof *in vitro* viral adaptation to cell culture also for Enterovirus A71 (92).

In this PhD research study, we report the promising anti-Coronavirus activity of Dispirotripiperazine-core derivatives, diazadispiroalkane.

Previously crowned as active molecules against HCMV (74), we investigated their activity against Coronaviruses, given the need to find new antiviral therapies in this pandemic period and to support the vaccination campaign.

As expected and in line with the literature knowledge (93) on the role of the Heparan Sulfate Proteoglycans in SARS-CoV-2 viral infection and their possible employ in disease treatment, none of molecules showed anti 229E activity, while several derivatives showed potent anti OC43 activity.

Different in vitro activities should be explained considering the differences between the two human viruses HCoV-229E (alphacoronavirus) and HCoV-OC43 (betacoronavirus). They bind different host cell receptors (human aminopeptidase N (hAPN) and 9-O-Acetyl-N-acetylneuraminic acid (Neu5,9Ac2), respectively and whereas OC43 is known to use HS, there is no evidence that heparan sulfates was required for 229E attachment and infection of target cells.

Among all investigated compounds, the derivative of diazadispiroalkane**236** was found to have a great broad-spectrum anti-β-Coronavirus activity with a cytotoxic profile in the high micromolar

range. To gain a detailed view of the efficacy of **236** against SARS-COV-2, a yield reduction test was performed showing a dose-dependent reduction in viral titer, even at low concentrations.

We first investigated the possibility that compound **236** acts directly on the viral particle leading to the inactivation of the virion, by means of a virucidal assay.

The results shown in Figure 8A showed that this compound does not directly target the virus.

The molecule's mechanism of action was confirmed by the adsorption and penetration assay and therefore occurs in the early stages of viral infection, as demonstrated when the compound was added during infection, while it is less effective on virus penetration into cells (Figure 9A and 9B).

In line with previous findings (94, 95) we can speculate that **236** avoids virus infection by binding to cell surface heparan sulfate glycosaminoglycans (HS) moieties.

CONCLUSIONS

During this PhD research, we identified PDSTP analogsas potent inhibitors of SARS-CoV-2. We showed that PDSTP derivatives are able to block *in vitro* the early phase of SARS-CoV-2 viral replication cycle. We also can speculate that viral adhesion to cellular proteoglycans of heparan sulfate is the main target of our molecules.

Due to their hypothesized mode of action, these compounds will be useful for prophylaxis as well as therapies.To further investigate and confirm the therapeutic potential of **236**, the next step would be to conduct *in vivo* experiments in animal models.

Future research may, in addition, help to widen molecule libraries for treating viruses for which currently there is no therapy.

REFERENCES

1. Reitz, S. R., & Trumble, J. T. (2002). Competitive displacement among insects and arachnids. Annual review of entomology, 47, 435–465. https://doi.org/10.1146/annurev.ento.47.091201.145227
2. White, R. J., &Razgour, O. (2020). Emerging zoonotic diseases originating in mammals: a systematic review of effects of anthropogenic land-use change. Mammal review, 10.1111/mam.12201. Advance online publication. https://doi.org/10.1111/mam.12201
3. Campos, F. S., de Arruda, L. B., & da Fonseca, F. G. (2021). Special Issue "Emerging Viruses 2020: Surveillance, Prevention, Evolution and Control". Viruses, 13(2), 251. https://doi.org/10.3390/v13020251
4. Nash D, Mostashari F, Fine A, et al. The outbreak of West Nile virus infection in the New York City area in 1999. *N Engl J Med*. 2001;344(24):1807-1814. doi:10.1056/NEJM200106143442401
5. Josseran, Loïc, et al. "Chikungunya disease outbreak, Reunion Island." Emerging infectious diseases vol. 12,12 (2006): 1994-5. doi:10.3201/eid1212.060710
6. Tandale BV, Sathe PS, Arankalle VA, et al. Systemic involvements and fatalities during Chikungunya epidemic in India, 2006. J ClinVirol. 2009;46(2):145-149. doi:10.1016/j.jcv.2009.06.027
7. Kariuki Njenga M, Nderitu L, Ledermann JP, et al. Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. J Gen Virol. 2008;89(Pt 11):2754-2760. doi:10.1099/vir.0.2008/005413-0
8. Monath TP, Vasconcelos PF. Yellow fever. J Clin Virol. 2015;64:160-173. doi:10.1016/j.jcv.2014.08.030
9. Duffy MR, Chen TH, Hancock WT, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360(24):2536-2543. doi:10.1056/NEJMoa0805715
10. Baize S, Pannetier D, Oestereich L, et al. Emergence of Zaire Ebola virus disease in Guinea. N Engl J Med. 2014;371(15):1418-1425. doi:10.1056/NEJMoa1404505
11. Lin YP, Shaw M, Gregory V, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci U S A. 2000;97(17):9654-9658. doi:10.1073/pnas.160270697

12. Claas EC, Osterhaus AD, van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus [published correction appears in Lancet 1998 Apr 25;351(9111):1292]. Lancet. 1998;351(9101):472-477. doi:10.1016/S0140-6736(97)11212-0
13. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans [published correction appears in N Engl J Med. 2009 Jul 2;361(1):102]. N Engl J Med. 2009;360(25):2605-2615. doi:10.1056/NEJMoa0903810
14. Ito T, Couceiro JN, Kelm S, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol. 1998;72(9):7367-7373. doi:10.1128/JVI.72.9.7367-7373.1998
15. Menachery VD, Yount BL Jr, Debbink K, et al. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence [published correction appears in Nat Med. 2016 Apr;22(4):446] [published correction appears in Nat Med. 2020 Jul;26(7):1146]. Nat Med. 2015;21(12):1508-1513. doi:10.1038/nm.3985
16. Drosten C, Günther S, Preiser W, et al. Identification of a novel coronavirus in patients with the severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1967-1976. doi:10.1056/NEJMoa030747
17. Peiris JS, Lai ST, Poon LL, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet. 2003;361(9366):1319-1325. doi:10.1016/s0140-6736(03)13077-2
18. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia [published correction appears in N Engl J Med. 2013 Jul 25;369(4):394]. N Engl J Med. 2012;367(19):1814-1820. doi:10.1056/NEJMoa1211721
19. Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. Clin Microbiol Rev. 2015;28(2):465-522. doi:10.1128/CMR.00102-14
20. Zhu N, Zhang D, Wang W, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382(8):727-733. doi:10.1056/NEJMoa2001017
21. Perlman S. 2020. Another Decade, Another Coronavirus. N Engl J Med. 2020; 382 (8), 760-2
22. Prusoff, W. H. 1959. Synthesis and biological activities of iododeoxyuridine, an analog of thymidine. Biochim. Biophys. Acta 32:295–296

23. Elion, G. B., P. A. Furman, J. A. Fyfe, P. de Miranda, L. Beauchamp, and H. J. Schaeffer. 1977. Selectivity of action of an antiherpetic agent, 9-(2- hydroxyethoxymethyl) guanine. Proc. Natl. Acad. Sci. USA 74:5716–5720
24. Schaeffer, H. J., L. Beauchamp, P. de Miranda, G. B. Elion, D. J. Bauer, and P. Collins. 1978. 9-(2-Hydroxyethoxymethyl) guanine activity against viruses of the herpes group. Nature 272:583–585
25. De Clercq E, Li G. Approved Antiviral Drugs over the Past 50 Years. Clin Microbiol Rev. 2016;29(3):695-747. doi:10.1128/CMR.00102-15
26. Ramírez-Olivencia G, Estébanez M, Membrillo FJ, Ybarra MDC. Use of ribavirin in viruses other than hepatitis C. A review of the evidence. Uso de ribavirina en virus distintos de la hepatitis C. Una revisión de la evidencia. EnfermInfecc Microbiol Clin (Engl Ed). 2019;37(9):602-608. doi:10.1016/j.eimc.2018.05.008
27. Stein DS, Creticos CM, Jackson GG, et al. Oral ribavirin treatment of influenza A and B. Antimicrob Agents Chemother. 1987;31(8):1285-1287. doi:10.1128/AAC.31.8.1285
28. Taylor M, Gerriets V. Acyclovir. In StatPearls. Treasure Island (FL): StatPearls Publishing; June 28, 2021
29. Malin JJ, Suárez I, Priesner V, Fätkenheuer G, Rybniker J. Remdesivir against COVID-19 and Other Viral Diseases. Clin Microbiol Rev. 2020;34(1):e00162-20. Published 2020 Oct 14. doi:10.1128/CMR.00162-20
30. Mercorelli B, Palù G, Loregian A. Drug Repurposing for Viral Infectious Diseases: How Far Are We?. Trends Microbiol. 2018;26(10):865-876. doi:10.1016/j.tim.2018.04.004
31. Drake JW, Holland JJ. Mutation rates among RNA viruses. Proc Natl Acad Sci U S A. 1999;96(24):13910-13913. doi:10.1073/pnas.96.24.13910
32. Hidalgo P, Valdés M, González RA. Molecular biology of coronaviruses: an overview of virus-host interactions and pathogenesis. BolMedHospInfantMex. 2021;78(1):41-58. English. doi: 10.24875/BMHIM.20000249. PMID: 33661875.
33. Woo PC, Lau SK, Lam CS, et al. Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J Virol. 2012;86(7):3995-4008. doi:10.1128/JVI.06540-11

34. Liu DX, Liang JQ, Fung TS. Human Coronavirus-229E, -OC43, -NL63, and -HKU1 (Coronaviridae). Encyclopedia of Virology. 2021;428-440. doi:10.1016/B978-0-12-809633-8.21501-X
35. Gildenhuys S. (2020). Expanding our understanding of the role polyprotein conformation plays in the coronavirus life cycle. The Biochemical journal, 477(8), 1479–1482. https://doi.org/10.1042/BCJ20200223
36. Fehr, A. R., & Perlman, S. (2015). Coronaviruses: an overview of their replication and pathogenesis. Methods in molecular biology (Clifton, N.J.), 1282, 1–23. https://doi.org/10.1007/978-1-4939-2438-7_1
37. Stertz, S., Reichelt, M., Spiegel, M., Kuri, T., Martínez-Sobrido, L., García-Sastre, A., Weber, F., & Kochs, G. (2007). The intracellular sites of early replication and budding of SARS-coronavirus. Virology, 361(2), 304–315. https://doi.org/10.1016/j.virol.2006.11.027
38. El-SahlyHM, Atmar RL, Glezen WP, Greenberg SB. Spectrum of clinical illness in hospitalized patients with “common cold” virus infection, Clin Infect Dis, 2000, vol. 31 (pg. 96-100)
39. Choi, WI., Kim, I.B., Park, S.J. et al. Comparison of the clinical characteristics and mortality of adults infected with human coronaviruses 229E and OC43. Sci Rep 11, 4499 (2021). https://doi.org/10.1038/s41598-021-83987-3
40. van der Hoek L, Pyrc K, Jebbink MF, et al. Identification of a new human coronavirus. Nat Med. 2004;10(4):368-373. doi:10.1038/nm1024
41. Woo PC, Lau SK, Chu CM, et al. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol. 2005;79(2):884-895. doi:10.1128/JVI.79.2.884-895.2005
42. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia [published correction appears in N Engl J Med. 2013 Jul 25;369(4):394]. N Engl J Med. 2012;367(19):1814-1820. doi:10.1056/NEJMoa1211721
43. Grange ZL, Goldstein T, Johnson CK, et al. Ranking the risk of animal-to-human spillover for newly discovered viruses [published correction appears in Proc Natl Acad Sci U S A. 2021 Sep 28;118(39):]. Proc Natl Acad Sci U S A. 2021;118(15):e2002324118. doi:10.1073/pnas.2002324118

44. Dijkman, R., Jebbink, M. F., Koekkoek, S. M., Deijs, M., Jónsdóttir, H. R., Molenkamp, R., Ieven, M., Goossens, H., Thiel, V., & van der Hoek, L. (2013). Isolation and characterization of current human coronavirus strains in primary human epithelial cell cultures reveal differences in target cell tropism. Journal of virology, 87(11), 6081–6090. https://doi.org/10.1128/JVI.03368-12
45. Liu, J., Li, Y., Liu, Q. et al. SARS-CoV-2 cell tropism and multiorgan infection. Cell Discov 7, 17 (2021). https://doi.org/10.1038/s41421-021-00249-2
46. Moriguchi, T., Harii, N., Goto, J., Harada, D., Sugawara, H., Takamino, J., Ueno, M., Sakata, H., Kondo, K., Myose, N., Nakao, A., Takeda, M., Haro, H., Inoue, O., Suzuki-Inoue, K., Kubokawa, K., Ogihara, S., Sasaki, T., Kinouchi, H., Kojin, H., … Shimada, S. (2020). The first case of meningitis/encephalitis associated with SARS-Coronavirus-2. International Journal of infectious diseases: IJID: official publication of the International Society for Infectious Diseases, 94, 55–58. https://doi.org/10.1016/j.ijid.2020.03.062
47. Kasereka, M.C., Hawkes, M.T. Neuroinvasive potential of human coronavirus OC43: case report of fatal encephalitis in an immunocompromised host. J. Neurovirol. 27, 340–344 (2021). https://doi.org/10.1007/s13365-020-00926-0
48. Arbour N, Ekandé S, Côté G, et al. Persistent infection of human oligodendrocytic and neuroglial cell lines by human coronavirus 229E. J Virol. 1999;73(4):3326-3337. doi:10.1128/JVI.73.4.3326-3337.1999
49. Hui KPY, Cheung MC, Perera RAPM, Ng KC, Bui CHT, Ho JCW, Ng MMT, Kuok DIT, Shih KC, Tsao SW, Poon LLM, Peiris M, Nicholls JM, Chan MCW. Tropism, replication competence, and innate immune responses of the coronavirus SARS-CoV-2 in human respiratory tract and conjunctiva: an analysis in ex-vivo and in-vitro cultures. Lancet Respir Med. 2020 Jul;8(7):687-695. doi: 10.1016/S2213-2600(20)30193-4. Epub 2020 May 7. PMID: 32386571; PMCID: PMC7252187.
50. Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellichio, C. B., Shapiro, L. H., Look, A. T., & Holmes, K. V. (1992). Human aminopeptidase N is a receptor for human coronavirus 229E. Nature, 357(6377), 420–422. https://doi.org/10.1038/357420a0
51. Wentworth DE, Holmes KV. Molecular determinants of species specificity in the coronavirus receptor aminopeptidase N (CD13): influence of N-linked glycosylation. J Virol. 2001;75(20):9741-9752. doi:10.1128/JVI.75.20.9741-9752.2001

52. Tortorici MA, Walls AC, Lang Y, et al. Structural basis for human coronavirus attachment to sialic acid receptors. Nat Struct Mol Biol. 2019;26(6):481-489. doi:10.1038/s41594-019-0233-y
53. Owczarek, K., Szczepanski, A., Milewska, A. et al. Early events during human coronavirus OC43 entry to the cell. Sci Rep 8, 7124 (2018). https://doi.org/10.1038/s41598-018-25640-0
54. Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N. H., Nitsche, A., Müller, M. A., Drosten, C., &Pöhlmann, S. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell, 181(2), 271–280.e8. https://doi.org/10.1016/j.cell.2020.02.052
55. Cagno, V., Tseligka, E. D., Jones, S. T., &Tapparel, C. (2019). Heparan Sulfate Proteoglycans and Viral Attachment: True Receptors or Adaptation Bias?. Viruses, 11(7), 596. https://doi.org/10.3390/v11070596
56. Yanmei Hu, Xiangzhi Meng, Fushun Zhang, Yan Xiang & Jun Wang (2021) The in vitro antiviral activity of lactoferrin against common human coronaviruses and SARS-CoV-2 is mediated by targeting the heparan sulfate co-receptor, Emerging Microbes & Infections, 10:1, 317-330, DOI: 10.1080/22221751.2021.1888660
57. Zhang, Q., Chen, C.Z., Swaroop, M. et al. Heparan sulfate assists SARS-CoV-2 in cell entry and can be targeted by approved drugs in vitro. Cell Discov 6, 80 (2020). https://doi.org/10.1038/s41421-020-00222-5
58. Yu M, Zhang T, Zhang W, Sun Q, Li H and Li J-p (2021) Elucidating the Interactions Between Heparin/Heparan Sulfate and SARS-CoV-2-Related Proteins—An Important Strategy for Developing Novel Therapeutics for the COVID-19 Pandemic. Front. Mol. Biosci. 7:628551. doi: 10.3389/fmolb.2020.628551
59. Chang, A., Masante, C., Buchholz, U. J., & Dutch, R. E. (2012). Human metapneumovirus (HMPV) binding and infection are mediated by interactions between the HMPV fusion protein and heparan sulfate. Journal of virology, 86(6), 3230–3243. https://doi.org/10.1128/JVI.06706-11
60. Patel, M., Yanagishita, M., Roderiquez, G., Bou-Habib, D. C., Oravecz, T., Hascall, V. C., & Norcross, M. A. (1993). Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. AIDS research and human retroviruses, 9(2), 167–174. https://doi.org/10.1089/aid.1993.9.167

61. Tan, C. W., Poh, C. L., Sam, I. C., & Chan, Y. F. (2013). Enterovirus 71 uses cell surface heparan sulfate glycosaminoglycan as an attachment receptor. Journal of virology, 87(1), 611–620. https://doi.org/10.1128/JVI.02226-12
62. Shukla, D., & Spear, P. G. (2001). Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. The Journal of clinical investigation, 108(4), 503–510. https://doi.org/10.1172/JCI13799
63. Weiss RJ, Esko JD, Tor Y. Targeting heparin and heparan sulfate protein interactions. Org Biomol Chem. 2017 Jul 21;15(27):5656-5668. doi: 10.1039/c7ob01058c. Epub 2017 Jun 27. PMID: 28653068; PMCID: PMC5567684
64. Mohamed, S., & Coombe, D. R. (2017). Heparin Mimetics: Their Therapeutic Potential. *Pharmaceuticals (Basel, Switzerland)*, *10*(4), 78. https://doi.org/10.3390/ph10040078
65. Mohan, C. D., Hari, S., Preetham, H. D., Rangappa, S., Barash, U., Ilan, N., Nayak, S. C., Gupta, V. K., Basappa, Vlodavsky, I., &Rangappa, K. S. (2019). Targeting Heparanase in Cancer: Inhibition by Synthetic, Chemically Modified, and Natural Compounds. *iScience*, *15*, 360–390. https://doi.org/10.1016/j.isci.2019.04.034
66. Schmidtke M, Riabova O, Dahse HM, Stelzner A, Makarov V. Synthesis, cytotoxicity and antiviral activity of N,N'-bis-5-nitropyrimidyl derivatives of dispirotripiperazine. Antiviral Res. 2002 Jul;55(1):117-27. doi: 10.1016/s0166-3542(02)00014-1. PMID: 12076756.
67. Schmidtke M, Karger A, Meerbach A, Egerer R, Stelzner A, Makarov V. Binding of an N,N'-bisheteryl derivative of dispirotripiperazine to heparan sulfate residues on the cell surface specifically prevents infection of viruses from different families. Virology. 2003 Jun 20;311(1):134-43. doi: 10.1016/s0042-6822(03)00166-1. PMID: 12832211.
68. Egorova A, Bogner E, Novoselova E, Zorn KM, Ekins S, Makarov V. Dispirotripiperazine-core compounds, their biological activity with a focus on broad antiviral property, and perspectives in drug design (mini-review). Eur J Med Chem. 2021;211:113014. doi:10.1016/j.ejmech.2020.113014
69. Novoselova, E. A., Riabova, O. B., Leneva, I. A., Nesterenko, V. G., Bolgarin, R. N., & Makarov, V. A. (2017). Antiretroviral Activity Of a NovelPyrimidyl-Di(Diazaspiroalkane) Derivative. *Acta naturae*, *9*(1), 105–107.
70. Adfeldt R, Schmitz J, Kropff B, et al. Diazadispiroalkane Derivatives Are New Viral Entry Inhibitors. Antimicrob Agents Chemother. 2021;65(4):e02103-20. Published 2021 Mar 18. doi:10.1128/AAC.02103-20

71. Imran, M., Kumar Arora, M., Asdaq, S., Khan, S. A., Alaqel, S. I., Alshammari, M. K., Alshehri, M. M., Alshrari, A. S., Mateq Ali, A., Al-Shammeri, A. M., Alhazmi, B. D., Harshan, A. A., Alam, M. T., &Abida (2021). Discovery, Development, and Patent Trends on Molnupiravir: A Prospective Oral Treatment for COVID-19. Molecules (Basel, Switzerland), 26(19), 5795. https://doi.org/10.3390/molecules26195795
72. Fischer, W., Eron, J. J., Holman, W., Cohen, M. S., Fang, L., Szewczyk, L. J., Sheahan, T. P., Baric, R., Mollan, K. R., Wolfe, C. R., Duke, E. R., Azizad, M. M., Borroto-Esoda, K., Wohl, D. A., Loftis, A. J., Alabanza, P., Lipansky, F., & Painter, W. P. (2021). Molnupiravir, an Oral Antiviral Treatment for COVID-19. medRxiv: the preprint server for health sciences, 2021.06.17.21258639. https://doi.org/10.1101/2021.06.17.21258639
73. Boras B, Jones RM, Anson BJ, et al. Discovery of a Novel Inhibitor of Coronavirus 3CL Protease for the Potential Treatment of COVID-19. Preprint. bioRxiv. 2021;2020.09.12.293498. Published 2021 Feb 12. doi:10.1101/2020.09.12.293498
74. Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J., & De Clercq, E. (1988). Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. Journal of virological methods, 20(4), 309–321. https://doi.org/10.1016/0166-0934(88)90134-6
75. Mendoza EJ, Manguiat K, Wood H, Drebot M. Two Detailed Plaque Assay Protocols for the Quantification of Infectious SARS-CoV-2. CurrProtoc Microbiol. 2020;57(1):ecpmc105. doi:10.1002/cpmc.105
76. Bogner, E., Egorova, A., & Makarov, V. (2021). Small Molecules-Prospective Novel HCMV Inhibitors. Viruses, 13(3), 474. https://doi.org/10.3390/v13030474)
77. Vijgen L, Keyaerts E, Moës E, et al. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. *J Virol*. 2005;79(3):1595-1604. doi:10.1128/JVI.79.3.1595-1604.2005
78. Selinka, H. C., Florin, L., Patel, H. D., Freitag, K., Schmidtke, M., Makarov, V. A., & Sapp, M. (2007). Inhibition of transfer to secondary receptors by heparan sulfate-binding drug or antibody induces noninfectious uptake of human papillomavirus. *Journal of virology*, *81*(20), 10970–10980. https://doi.org/10.1128/JVI.00998-07
79. Paeschke R, Woskobojnik I, Makarov V, Schmidtke M, Bogner E. DSTP-27 prevents entry of human cytomegalovirus. *Antimicrob Agents Chemother*. 2014;58(4):1963-1971. doi:10.1128/AAC.01964-13

80. Dyer, A. P., Banfield, B. W., Martindale, D., Spannier, D. M., &Tufaro, F. (1997). Dextran sulfate can act as an artificial receptor to mediate a type-specific herpes simplex virus infection via glycoprotein B. Journal of virology, 71(1), 191–198. https://doi.org/10.1128/JVI.71.1.191-198.1997
81. Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J., & De Clercq, E. (1988). Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. Proceedings of the National Academy of Sciences of the United States of America, 85(16), 6132–6136. https://doi.org/10.1073/pnas.85.16.6132
82. Chen, Y., Liu, Q., & Guo, D. (2020). Emerging coronaviruses: Genome structure, replication, and pathogenesis. *Journal of medicalvirology*, *92*(4), 418–423. https://doi.org/10.1002/jmv.25681
83. Cui, J., Li, F. & Shi, ZL. Origin and evolution of pathogenic coronaviruses. *NatRevMicrobiol* **17,** 181–192 (2019). https://doi.org/10.1038/s41579-018-0118-9
84. Weiss, Susan R, and Julian L Leibowitz. "Coronavirus pathogenesis." *Advances in virus research* vol. 81 (2011): 85-164. doi:10.1016/B978-0-12-385885-6.00009-2
85. Desforges M, Le Coupanec A, Stodola JK, Meessen-Pinard M, Talbot PJ. Human coronaviruses: viral and cellular factors involved in neuroinvasiveness and neuropathogenesis. *Virus Res*. 2014;194:145-158. doi:10.1016/j.virusres.2014.09.011
86. Morfopoulou S, Brown JR, Davies EG, et al. Human Coronavirus OC43 Associated with Fatal Encephalitis. *N Engl J Med*. 2016;375(5):497-498. doi:10.1056/NEJMc1509458
87. Sidebottom, D. B., Smith, D. D., & Gill, D. (2021). Safety and efficacy of antivirals against SARS-CoV-2. *BMJ (Clinicalresearch ed.)*, *375*, n2611. https://doi.org/10.1136/bmj.n2611
88. Mahase E. (2021). Covid-19: Pfizer's paxlovid is 89% effective in patients at risk of serious illness, company reports. *BMJ (Clinicalresearch ed.)*, *375*, n2713. https://doi.org/10.1136/bmj.n2713
89. Welte, T., Ambrose, L. J., Sibbring, G. C., Sheikh, S., Müllerová, H., & Sabir, I. (2021). Current evidence for COVID-19 therapies: a systematic literature review. *European respiratory review : an official journal of the European Respiratory Society*, *30*(159), 200384. https://doi.org/10.1183/16000617.0384-2020

90. Bobrowski, T., Chen, L., Eastman, R. T., Itkin, Z., Shinn, P., Chen, C. Z., Guo, H., Zheng, W., Michael, S., Simeonov, A., Hall, M. D., Zakharov, A. V., &Muratov, E. N. (2021). Synergistic and Antagonistic Drug Combinations against SARS-CoV-2. *Molecular therapy : the journal of the American Society of Gene Therapy*, *29*(2), 873–885. https://doi.org/10.1016/j.ymthe.2020.12.016
91. Szczepanski, Artur et al. "Canine Respiratory Coronavirus, Bovine Coronavirus, and Human Coronavirus OC43: Receptors and Attachment Factors." *Viruses* vol. 11,4 328. 5 Apr. 2019, doi:10.3390/v11040328
92. Chamberlain, Kyle et al. "Identification of a novel cell culture adaptation site on the capsid of foot-and-mouth disease virus". *The Journal of general virology* vol. 96,9 (2015): 2684-2692. doi:10.1099/jgv.0.000222
93. De Pasquale, V.; Quiccione, M.S.; Tafuri, S.; Avallone, L.; Pavone, L.M. Heparan Sulfate Proteoglycans in Viral Infection and Treatment: A Special Focus on SARS-CoV-2. *Int. J. Mol. Sci.* **2021**, *22*, 6574. https://doi.org/10.3390/ijms22126574
94. Tan CW, Chan YF. Enterovirus 71 receptors: promising drug targets?. *Expert Rev Anti Infect Ther*. 2013;11(6):547-549. doi:10.1586/eri.13.47
95. Compton, T., Nowlin, D. M., & Cooper, N. R. (1993). Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology*, *193*(2), 834–841. https://doi.org/10.1006/viro.1993.1192