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Studies of broad-spectrum inhibitors against main protease of SARS-CoV-2 and other Coronaviruses

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Abstract

Coronaviruses have caused three large outbreaks in the past century. The most recent one, also still ongoing, is represented by the SARS-CoV-2/Covid-19 pandemic. Efforts have been taken to develop efficient vaccines and antivirals and one of the major virus-based targets in drug development is represented by the main protease of these viruses. Main proteases are proteins (cysteine hydrolases) with high level of conservation among different coronaviruses and have an important role in the virus life cycle. Due to the need of developing broad-spectrum antivirals against Coronaviruses, this study aimed to set up a CPE-based assay for testing compounds against the main protease of human coronavirus 229E. An optimized $TCID_{50}$ protocol was established by using MRC-5 cells, at a density of $1x10^4$ cells/ml with a 3h incubation prior infection with a concentration of 10^{-1} of HCoV-229E. The cell viability was assessed through MTT assay. Using reference compounds, with previously demonstrated antiviral potency against the main protease of different coronaviruses (GC-376, Nirmatrelvir), the efficiency of the conceived assay was validated (GC-376 EC_{50} = 1.24 μM; Nirmatrelvir Ec_{50} = 0.72 μM). Compound 19 was proved to also be active against the main protease of HCoV-229E (EC_{50} = 0.22 μM), and together with previous findings, it was concluded that this compound has a broad-spectrum activity. Newly developed compounds MP17 and MP19 were also demonstrated to be efficient against HCoV-229E. As a future perspective, further investigations of these compounds should take place for the identification of EC_{50} values.

Popular science summary

How a broad-spectrum antiviral against Coronaviruses can be found?

Coronaviruses have gained a lot of attention during the past years, since they caused the most recent pandemic – the SARS-CoV-2 pandemic. However, these viruses have also caused other large outbreaks since 2000: SARS and MERS. Coronaviruses represent a threat to the human population and due to their impact on the society, great efforts were made to develop effective vaccines and treatments. Several drugs have already been released on the market, but there is still a need to have a broad-spectrum antiviral drug against coronaviruses. A good target candidate is the main protease, which is an important protein of these viruses due to its implication in the coronavirus life cycle.

This present study succeeded to design an assay with cells that would allow testing of antiviral potency of different compounds against the main protease of a human coronavirus causing common cold (HCoV-229E). Furthermore, a number of compounds were tested and their ability to inhibit the HCoV-229E infection was demonstrated.

When it comes to the experimental approach, human fibroblast lung cells (MRC-5) were chosen and the optimal conditions for them to grow and to be infected with HCoV-229E were found. The next step of this project was represented by testing the antiviral potency of various compounds. To validate that the designed assay was successful, compounds that were previously demonstrated to be active against the main protease of coronaviruses were used. One of them was **GC-376**, a compound developed against a coronavirus causing infectious peritonitis in cats, also with shown antiviral activity against human coronaviruses, such as SARS, SARS-CoV-2 and even HCoV-229E. The other compound was **Nirmatrelvir**, which is a component of the novel drug Paxlovid, this being approved by FDA as a treatment for severe cases of COVID-19. Validation of the assay success was based on EC_{50} values, which are the half maximal effective concentrations of tested compounds or drugs.

Then, compound **19** was tested. This compound was recently found to be active against SARS-CoV-2, SARS, and MERS. In this project, its antiviral potency was demonstrated also against HCoV-229E. Thereby, the broad-spectrum profile of this compound against Coronaviruses was revealed. In addition to these, novel compounds were obtained through a collaboration with Oscar Verho (Department of Medicinal Chemistry, UU,) Martin Moche (Protein Science Facility (PSF), KI) and Kristian Sandberg (SciLifeLab DDD, UU). When these compounds were investigated against the HCoV-229E infection, two of these were able to inhibit changes in the cell's morphology: **MP17** and **MP19**.

To conclude, this study achieved its aims expanding the current knowledge in the drug development against Coronaviruses. One of the benefits could be the disclosed broad-spectrum profile of the compound **19**, but also the identification of the antiviral potency of the newly developed compounds. The latter leads to future perspectives, i.e., finding the optimal concentrations of these compounds effective against infections caused by different coronaviruses.

Key words: human coronavirus 229E, SARS-CoV-2, main protease, $TCID_{50}$, nirmatrelvir, GC-376, compound 19

Introduction

Coronavirus outbreaks, origin, and diversity

The 21st century was marked by three important outbreaks caused by Coronaviruses (CoVs): severe acute respiratory syndrome (SARS-CoV) in 2002-2003 localized in Foshan, Guangdong province of China (Zhong et al., 2003), Middle East respiratory syndrome-related coronavirus (MERS-CoV) in 2012, identified in Saudi Arabia (Zumla et al., 2015), and from the end of 2019 to present time, SARS-CoV-2, originating from a seafood market in Wuhan, China (Zhu et al., 2019).

However, the story of CoVs began in 1937, when the initial coronavirus was described, namely infectious bronchitis virus (IBV), isolated from chicken embryos. During the years, multiple CoVs were identified in various species of animals, from wild or farm animals to pets (Ludwig & Zarbock, 2020).

According to the International Committee of Taxonomy of Viruses (ICTV), Coronaviruses belong to *Coronavirinae* subfamily of *Coronaviridae* family in the *Nidovirales* order. If taking into consideration phylogenetic relationships and genomic assemblies, *Coronavirinae* subfamily has been divided into four genera: *Alpha*-, *Beta*-, *Gamma*- and *Delta-coronavirus* (Cui et al., 2019). *Alpha*- and *Beta-coronaviruses* cause infections only in mammals, leading to respiratory diseases in humans or gastroenteritis in animals, whereas *Gamma*- and *Delta-coronaviruses* mostly infect birds and sometimes mammals (Cui et al., 2019).

There are seven types of Coronaviruses known to cause infections in humans. Most pathogenic strains belong to *Beta-CoV* family: SARS-CoV, MERS-CoV and SARS-CoV-2, leading to severe respiratory syndrome (Cui et al., 2019). The other four strains of human coronaviruses: HCoV-229E, -NL63, -OC43 and -HKU1, are known to be causative agents of mild upper respiratory diseases. These viruses are part of different genera: 229E and NL63 are recognized as alphacoronaviruses, whereas OC43 and HKU1 are betacoronaviruses (CDC, n.d.).

When it comes to the origin of the human coronaviruses, current sequence databases demonstrated that they all have zoonotic origins (Holmes et al., 2021). Some strains are thought to have originated from bats, such as SARS, MERS, SARS-CoV-2, NL-63 and 229E, whereas OC43 and HKU1 have a probable origin in rodents (Forni et al., 2017).

Coronaviruses genome

Coronaviruses have a linear single-stranded, positive sense RNA genome, being the largest among the RNA viruses: 27-32 kb and it carries a 5' methylguanosine cap and a 3' poly(A) tail (Fehr & Perlman, 2015). CoV virion is described as a spherical enveloped particle, having a size between 75-160 nm. Its surface has a specific feature represented by spike proteins resembling the Sun's corona, thus explaining the virus family name (Fehr & Perlman, 2015).

Their genome encloses 6-10 open reading frames (ORFs), the number being determined by individual viral strains. Genes that are coding virion structural proteins and group-specific nonstructural proteins (nsps) are contained in one-third of the genome (Denison & Becker, 2011). Four major structural proteins can be seen in all CoVs (Schoeman & Fielding, 2019), arranged in the indicated order: spike protein (S), small envelope protein (E), membrane protein (M) and nucleocapsid protein (N). Furthermore, a fifth protein can be found in some coronaviruses (betacoronaviruses): hemagglutinin-esterase (HE) (Denison & Becker, 2011).

Besides the gene order, which is conserved, homologous recombination can often take place in coronaviruses (de Klerk et al., 2022). Additionally, CoVs are known for their less elevated mutation rate compared to other RNA viruses justified by the presence of nsp14. Its subunit has proof-reading ability and is connected to nsp12, i.e., RNA-dependent-RNA-polymerase (RdRp) subunit (Smith and Denison, 2013). The RdRp and its linked subunits play the essential role in duplication of genetic information (Duffy et al., 2008).

Consequently, these mentioned properties of CoVs support their impressive ability of creating genetic diversity, justifying their capacity to infect various species (Ludwig & Zarbock, 2020).

Coronaviruses pathogenesis

It is known that coronaviruses spike proteins can recognize and bind to a multitude of receptors. SARS, SARS-CoV-2 and NL63 attach to a metalloproteinase named angiotensin-converting enzyme 2 (ACE2) (Shang et al., 2020). HCoV-229E uses the human aminopeptidase (hAPN) as receptor, which is a metalloprotease found on the cell surface (Yeager et al., 1992), whereas MERS utilizes dipeptidyl peptidase 4 (DPP4), which is a serine protease (Raj et al., 2013).
The main consequence of CoV infection is represented by respiratory tract infections (RTIs). The severity is varied, since one could get from a common cold to bronchiolitis, pneumonia or even gastroenteritis (Denison & Becker, 2011). This wide range of diseases could be justified by the dissimilar cell tropism of each Coronavirus. SARS-CoV and SARS-CoV-2 have been showed to infect the ciliated, but also alveolar type 2 cells localized across both the upper and lower respiratory tract (Sungnak et al., 2020). MERS-CoV was demonstrated to be able to infect non-ciliated bronchial epithelial cells, bronchiolar and alveolar (type 1 and type 2) epithelial cells, but also endothelial cells from pulmonary vessels (Meyerholz et al., 2016). When it comes to HCoV-229E, it was suggested that most probably the natural infection is initiated by the infection of the ciliated epithelial cells along the nasopharynx (Liu et al., 2021). Since all coronaviruses are causative agents of different RTIs, the modes of transmission are mainly represented by direct contact with an infected individual through droplets, aerosols, fomites and even body fluids, i.e., saliva (Zhou et al., 2021).

Human coronavirus 229E (HCoV-229E)

HCoV-229E strain is one of the first coronaviruses in humans that has been described, in 1960s, together with HCoV-OC43 (Ludwig & Zarbock, 2020). It is suggested that HCoV-229E is originating from the African hipposiderid bats using camelids as intermediate hosts (Liu et al., 2021). The two strains along with HCoV-NL63 and HCoV-HKU1 are recognized as endemic viruses, circulating in humans all over the world. Generally, they lead to quite mild illnesses of the upper and lower respiratory tract, being documented that these HCoV are responsible for approximately one third of the human common colds (Ludwig & Zarbock, 2020). The infections range from asymptomatic to more severe outcomes. For example, the disease might progress to acute respiratory failure, especially in immunocompromised individuals, children, or people with preexisting conditions, i.e., pulmonary diseases (Corman et al., 2019).
One infected with HCoV-229E can exhibit typical common cold symptoms, such as headache, nasal discharge, sneezing, sore throat, and general malaise. Other patients (10-20%), could also display signs of fever and cough (Su et al., 2016). HCoV-229E has an incubation time of roughly 2-5 days, succeeded by the disease itself which has a duration of 2-18 days (Liu et al., 2021). Moreover, previous data suggests that symptoms of HCoV-229E have a peak generally on day 3 or 4 of disease and have a self-limiting behavior (Poutanen, 2012). Interestingly, HCoV-229E infections are difficult to be clinically distinguished from other respiratory tract infections, for example influenza A virus or rhinovirus (Su et al., 2016).

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

As aforementioned, SARS-CoV-2 is one of the most pathogenic Coronavirus and is the causative agent of the still ongoing pandemic. In the beginning, the virus which caused sudden and severe cases of pneumonia was called novel Coronavirus 2019 (nCoV-2019) (Li et al., 2020). Later on, it has been revealed that nCoV-2019 shares 79.5% with the SARS-CoV genome sequence, thus being renamed SARS-CoV-2 (Ludwig and Zarbock, 2020). The disease caused by it also received a name: coronavirus disease 2019 (COVID-19) (Xu et al., 2020).

The infection is transmitted from person to person, via droplets, but also through aerosols or fomites (van Doremalen et al., 2020). SARS-CoV-2 has an incubation period of 3-7 days (range 2-14 days) followed by the respiratory illness, COVID-19 (Huang et al., 2020). The disease can be characterized by a wide variety of symptoms, from flu-like signs (e.g., fever, chills, cough, muscle/body aches etc.) to loss of smell/taste, shortness of breath, difficulties in breathing or gastro-intestinal symptoms, such as diarrhea, nausea, vomiting (CDC, n.d.). Furthermore, the course of this disease has a broad spectrum, since it can be asymptomatic/pre-symptomatic, mild, moderate, severe, or critical (Binns et al., 2020). The critical cases of COVID-19 are usually patients displaying life-threatening conditions (i.e., acute respiratory distress syndrome (ARDS), septic shock and/or multiple organ dysfunction), ultimately leading to death (Wu & McGoogan, 2020). ARDS and multiple organ failure are generally caused by the cytokine storm syndrome (Villar et al., 2019), an excessive production of pro-inflammatory cytokines, such as IFN-γ and TNF-α (Del Valle-Mendoza et al., 2022).

Vaccines, treatments & drug targets

A number of COVID-19 vaccines were developed, approved, or authorized, such as Pfizer-BioNTech and Moderna (mRNA vaccines), Novavax (protein subunit vaccine) and Johnson & Johnson's Janssen (viral vector vaccine) (CDC, n.d.). However, observational studies have indicated that these vaccines are offering a high protection only against a severe disease caused by SARS-CoV-2, hospitalization, and death (ECDC, 2022), thus leading to the need of an effective defense for those acquiring the infection.

When developing a drug against SARS-CoV-2, multiple virus-based targets are considered, for example N gene, RdRp or the main protease (M^{pro}), also termed the 3C-like protease ($3CL^{pro}$) (Gil et al., 2020). Currently, there are several drugs approved for treatment of individuals diagnosed with severe COVID-19, namely Paxlovid, Remdesivir or Molnupiravir (Cascella et al., 2022). Paxlovid is the first antiviral drug acting specifically against COVID-19 that has been approved by FDA. It is a combination of ritonavir and nirmatrelvir; ritonavir is a known HIV-1 protease inhibitor, but it also inhibits the cytochrome P450 (CYP) 3A and can thereby elevate the nirmatrelvir effect against SARS-CoV-2 (Najjar-Debbiny et al., 2023). Thus, nirmatrelvir represents a M^{pro} inhibitor, restricting its enzymatic activity (Dawood, 2022). Remdesivir, another drug used in COVID-19 emergency cases, is described as being a nucleoside analog. This drug behaves as a competitive inhibitor of the RdRp (Frediansyah et al., 2021) and it is able to evade the proofreading mechanisms of the viruses leading to a decreased viral RNA production (Scavone et al., 2020). Molnupiravir is a broad-spectrum oral antiviral drug which is also directly interfering with the RdRp. At first, this antiviral agent was produced as a treatment option for influenza and alphaviruses (i.e., Eastern/Western equine encephalitis viruses) (Cascella et al., 2022). Later on, a meta-analysis study showed an impressive reduction of mild to moderate COVID-19 cases that could progress to more severe cases resulting in hospitalization or death (Singh et al., 2021).

Based on the mentioned facts, the M^{pro} and RdRp of CoVs are highly pertinent virus-based targets, and this is proven by the approved drugs already released on the market.

M^{pro}/$3CL^{pro}$ as drug target

M^{pro}, as afore mentioned, represents one of the virus-based targets when attempting to develop new drugs against CoVs. Main proteases are classified as cysteine hydrolases, with a high level of conservation, competent of cleavage of the two viral polyproteins (pp1ab and pp1a) at eleven sites, leading to production of 16 functional proteins (Qiao et al., 2021).

When it comes to the structural characteristics of the M^{pro}, in its active form it is found as a dimer. The monomers have a reduced enzymatic activity, and they comprise three domains: I, II and III (Abe et al., 2022). The location of the catalytic site corresponding to $3CL^{pro}$ is found

at the junction of domains I and II (Yang et al., 2003) and its catalytic dyad consists of the coordinated amino acid residues Cys145 and His41 (Chen et al., 2008).
Based on the idea that the M^{pro} is highly involved in the replication of coronaviruses, they were validated as potential targets for the development of broad-spectrum antiviral drugs against CoV (Zhang et al., 2020). Furthermore, there is no similarity when comparing to the cysteine proteases found in humans, meaning that it is suitable and possible to produce specific inhibitors for this protease. Since a very low inhibitory effect would be noticed in human proteases, any possible side effects that might be caused by these are diminished (Hu et al., 2022). Taking these facts into consideration, various compounds were developed, for example compound 19 (Luttens et al., 2022) or Pfizer's clinical candidate, PF-07321332, nowadays known as Nirmatrelvir (Owen et al., 2021). Another example is Ensitrelvir fumaric acid, inhibitor of SARS-CoV-2 M^{pro}, discovered and developed in Japan. By using Ensitrelvir, viral replication should be prevented and as expected, this drug demonstrated antiviral efficacy when it was tested both *in vitro* and *in vivo* animal studies (Unoh et al., 2022).
Summing up all the mentioned facts, there is an increased need, but also an interest for developing anti-viral agents against Coronaviruses (targeting the M^{pro}). However, more research in this direction has to happen, so an inhibitor with broad-spectrum activity could be released on the market. In this way, a new possible outbreak could be avoided, and the risks associated with it diminished.

Aim of the study

Coronaviruses caused three notable outbreaks and during the ongoing pandemic of SARS-CoV-2, multiple vaccines and several drugs were produced (ECDC, 2022). As outlined above, one viral-based target of interest in drug development is the main protease, a protein with a major role in the virus life cycle (Zhang et al., 2020).
Consequently, the aim of this study was represented by setting up a cell-based assay that would allow the investigation of the antiviral potency of compounds against HCoV-229E. For this purpose, compounds that were previously demonstrated to be active against M^{pro} of different CoVs (SARS, MERS, SARS-CoV-2) were tested against the infection with HCoV-229E. If successful, this study could answer important questions related to the development of broad-spectrum inhibitors against CoVs.

Materials and methods

Similarity of M^{pro} between CoVs

Using the NCBI database, the complete genomes of the four viruses were found: **HCoV-229E** – RefSeq NC_002645.1, **SARS Sin2679** - GenBank: AY283796.1, **SARS-CoV-2** Wuhan-HU-1 – RefSeq NC_045512.2, **MERS** – RefSeq NC_019843.3. Following the identification of the complete genomes, the nucleotides (nt) corresponding to the M^{pro} were found for all genomes. This was followed by a Multiple Sequence Alignment to identify the similarity between the four proteases, which was obtained from the Percent Identify Matrix.

Cell culture

MRC-5 cell line (human fibroblast lung cells, ATCC® (CCL-171™)) was grown and maintained in Dulbecco's Modified Eagle Medium - DMEM (Gibco, catalog no. 11965092) supplemented with of 10% fetal bovine serum - FBS (Gibco, catalog no. 10500064) and 1% penicillin-streptomycin 10000 U/mL^{-1} (Gibco catalog no. 15140148). When cells reached confluency, they were subcultured in a 1:1 ratio.

For counting the cells, a mixture in 1:1 ratio of resuspended cells and Trypan-blue was prepared and transferred to a hemocytometer, counting the cells under the microscope. To obtain a specific number of cells per ml, the following formula has been used: $\frac{desired\ number\ of\ cells \times volume\ needed}{number\ of\ counted\ cells}$.

Virus propagation

When MRC-5 cells reached 70-80% confluency, the infection media was prepared. In a volume of 27 ml of DMEM 2% FBS, 500 µl of HCoV-229E (VR-740™, ATCC, 2nd passage) were added. Following the washing step of cells, 5 ml of infection media were transferred per flask, followed by 1-hour incubation at 37°C, 5% CO_2.The flasks were shaken every 15 minutes. A final volume of 9 ml was reached by adding DMEM 10% FBS. Then the plates were placed again in the incubator for 5 days.

To harvest the virus, the media from flasks was transferred in a 50 ml falcon tube, which was centrifuged at 3000 g x10 minutes at room temperature. The virus was aliquoted (500 µl in 2 ml tubes) and stored in the -80°C freezer.

Plaque assay

To be able to count the infectious particles present in the obtained viral stock, a plaque assay was conducted. MRC-5 cells were seeded in 6-well plates, each well containing 1 ml of DMEM 2% FBS and 1 ml cells. The overlay consisted in mixing 2x MEM (Gibco, catalog no. 11935046) and agar in 1:1 volume ratio. Different FBS and agar concentrations were tested: 2% FBS and 0.5% agar, 2% FBS and 0.75% agar, 8% FBS and 0.75% agar. For the HCoV-229E infection, a 1:10 serial dilution was prepared, ranging from 10^{-1} to 10^{-5}. The current media from the wells was replaced with 100 µl of infection media or 100 µl of DMEM 2% FBS for the mock-infected well. The plates were placed in the incubator at 37°C, 5% CO_2 for 1 hour, being shaken every 10 minutes. Then, the overlay was added (2 ml). When the agar solidified, the plates were placed in the incubator for 5 days. Lastly, 2 ml of neutral red solution (3%) were added per well, followed by an incubation time of 2-4 hours. After this, the plates were checked for plaques.

50% Tissue Culture Infectious Dose ($TCID_{50}$)

For the quantification and assessment of the infectivity of obtained stock of HCoV-229E in MRC-5 cells, a $TCID_{50}$ experiment was carried out. For this purpose, 10.000 cells/well were seeded in a 96-well plate (100 µl of cell solution per well). To prepare the infection media, a 1:10 serial dilution was conducted, by adding in a 1.5 ml Eppendorf tube 100 µl virus and 900 µl of DMEM 2% FBS, obtaining viral concentrations from 10^{-1} to 10^{-9}. When the cells attached to the wells, the old media was replaced with 100 µl of the infection media. Lastly, the plate was incubated at 37°C, 5% CO_2 for 5 days to allow the cytopathic effects (CPE) development.

HCoV-229E CPE-based antiviral assay

The potency of Compound **19** and **Nirmatrelvir (PF-07321332)** was tested at final concentrations that ranged from 2.5 µM to 0.019 µM. For compounds **GC-376** and **MP9,** the final concentrations tested ranged from 20 µM to 0.156 µM, whereas the compounds **MP11**, **MP16**, **MP17**, **MP18**, **MP19** and **OVE-002 (12-1)** were tested at final concentrations of 20 µM and 10 µM. For each concentration tested of the compounds **19**, **Nirmatrelvir**, **GC-376** and **MP9** triplicates in minimum two separate experiments were carried out.

Newly developed compounds (**MP11**, **MP16**, **MP17**, **MP18**, **MP19** and **OVE-002 (12-1)**) were obtained through collaboration with Oscar Verho (Department of Medicinal Chemistry, UU,) Martin Moche (Protein Science Facility (PSF), KI) and Kristian Sandberg (SciLifeLab

DDD, UU). The interest regarding these consisted only in finding out their ability to inhibit the CPE formation caused by the HCoV-229E infection, thus only one experiment was performed, including triplicates as well for each concentration. MRC-5 cells were seeded in 96-well plates (10.000 cells/well) in a final volume of 100 µl of DMEM 10% FBS and placed in the incubator (37°C, 5% CO_2) for 4 hours. Meanwhile, aliquots of the compounds in dimethyl sulfoxide (DMSO) were serially diluted (1:2 ratio) in DMSO, followed by second dilution in DMEM 2% FBS to 4x working solutions of the needed final concentrations. When the cells attached to the wells, the preexisting cell media was removed and replaced with 50 µl of new DMEM (2% FBS). Then, the cells were infected with 25 µl of HCoV-229E (at a concentration of 10^{-1}) and treated with 25 µl of the desired compound found in a concentration 4x higher. In this way, the compounds were diluted once again (1:4) reaching the final concentration of interest and the final volume in the well reached 100 µl. Each plate contained cells infected-treated, uninfected-treated, but also cells only infected and uninfected-untreated, used as controls; all the wells contained DMSO (0.2%, v/v).

MTT cell viability assay

When CPE was noticed in the cells, following 5 days of incubation, the MTT assay was performed. This consisted in adding 10 µl of a MTT staining solution (5 mg/ml) in PBS (Sigma-Aldrich, M2128). After 1,5 hour of incubation, the established formazan crystals were solubilized with 100 µl of a solution of 10% sodium dodecyl sulfate (SDS), 0.01 M HCl. This was succeeded by overnight incubation. By using a Tecan Infinite M200 PRO plate reader (Tecan Trading AG, Switzerland) the optical density (OD) was read at 570 and 690 nm. Resulted OD readings at the two wavelengths were subtracted and the obtained values have been normalized to the controls. To determine the EC_{50}, the non-linear regression analysis was conducted utilizing GraphPad Prism (v9.5.1) (GraphPad Software, La Jolla California, USA).

Neutral red uptake assay

Another cell viability assay was also performed, being represented by the neutral red uptake assay. Five days post infection (pi), the cell media was removed, and the cells were stained with 100 µl of neutral red staining solution with DMEM 2% FBS (50 µg/ml). Following 2h of incubation, the wells were washed with 200 µl of PBS. When the plates were completely dry, 100 µl of de-staining solution was added (70% EtOH, 29% deionized water, 1% glacial acetic acid). Then, the plates were placed on a microtiter plate at 300 rpm for 15 minutes and OD values were read at 540 nm using the Tecan Infinite M200 PRO plate reader (Tecan Trading AG, Switzerland).

Crystal violet staining method

A third cell viability assay was tested: the crystal violet staining method. Following 5 days pi, current cell media from the wells was discarded, and the cells were washed with 100 µl PBS. Then, 50 µl of crystal violet staining solution (0.5%) in distilled water and methanol were added. After 20 minutes, the cells were washed twice with 200 µl PBS and left to air dry. The cells were then de-stained with 200 µl methanol and following 20 minutes of incubation at room temperature, the OD was read at 570 nm using the Tecan Infinite M200 PRO plate reader (Tecan Trading AG, Switzerland).

Results

Similarity of M^{pro} between CoVs

The multiple sequence alignment, more specifically the Percent Identify Matrix performed on the sequences of the four CoVs indicated that HCoV-229E M^{pro} has a similarity of 49.34% with MERS M^{pro}, 40.53% with SARS M^{pro} and 41.2% with SARS-CoV-2 M^{pro} (Table 1).

Similarities of main protease sequnces between different Coronaviruses

M^{pro} sequences	HCoV-229E	MERS-CoV	SARS-CoV	SARS-CoV-2
HCoV-229E	100.00%	49.34%	40.53%	41.20%
MERS-CoV	49.34%	100.00%	51.82%	50.83%
SARS-CoV	40.53%	51.82%	100.00%	96.08%
SARS-CoV-2	41.20%	50.83%	96.08%	100.00%

Table 1. Values from the Percent identity matrix obtained after running the Multiple Sequence Alignment to identify how similar is the M^{pro} of HCoV-229E to the M^{pro} of the MERS, SARS Sin2679 and SARS-CoV-2.

Plaque assay & $TCID_{50}$ on the obtained virus stock

The number of infectious particles found in the HCoV-229E after performing the plaque assay (2% FBS, 0.5% agar) was found to have a value of $1.3x10^3$ PFU/ml.

Further assessment and quantification of virus stock infectivity through $TCID_{50}$ method was calculated based on several distinct methods. The first method was called Method 1 – Qualitative, as it was based on the observation of the plate under the microscope. The infected wells with CPE have been distinguished from the wells that did not develop CPE, even if they were infected. This method indicated that 50% of the wells were infected at the 10^{-5} viral concentration.
The second method, named Method 2 – Quantitative was based on optical density (OD) values of the same plate measured using the plate reader. Since two readings were obtained, a difference was calculated between the two values, followed by calculating the average of the OD values of the control wells (not infected cells). Then, standard deviation (SD) of the average was calculated. The OD values smaller than the value obtained after subtracting 3*SD from average were considered to represent infected wells. Based on this analysis, the $TCID_{50}$ was observed to be $10^{-3.}$

To have an improved visualization on the differences in the obtained $TCID_{50}$ values, a bar chart was created using the GraphPad software (Figure 1A). Since a clear difference has been noticed, a third method was needed to calculate the $TCID_{50}$, which was represented by the MTT cell viability assay. This method indicated that the $TCID_{50,}$ meaning the virus concentration that produced CPE in 50% of the wells, had a value of $10^{-2.9}$ (value extracted from GraphPad) (Figure 1B). For a better understanding of which $TCID_{50}$ assay setup worked better, a comparison between the three used methods has been done. As it can be observed in Figure 1C, the quantitative method (based on OD values) and the MTT assay indicated a similar result, whereas the qualitative method (observation of the wells under the microscope) had a slightly different outcome.

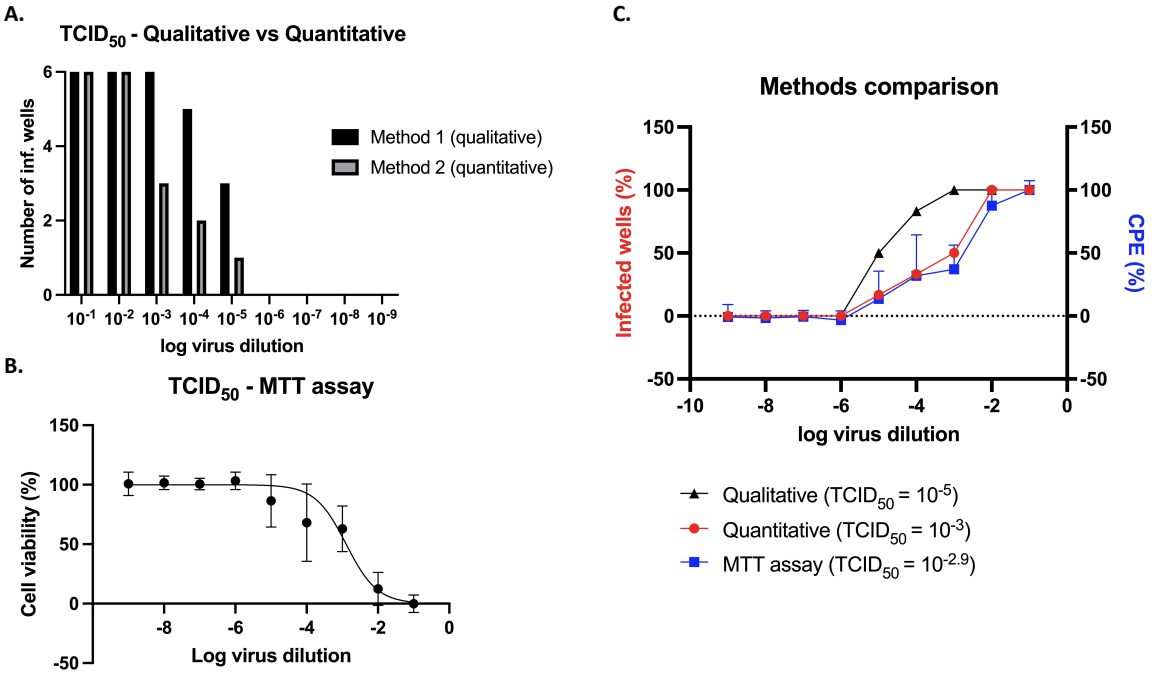

Figure 1. (A) $TCID_{50}$ values calculated based on two different methods: qualitative method (observation of the wells under the microscope; infected well vs not infected well) and quantitative method (Infected well if OD value ≤ average OD of control cells ± 3*SD). (B) MTT cell viability assay indicating the development of 50% CPE in the analyzed wells containing MRC-5 cells infected with HCoV-299E varying from 10^{-1} to 10^{-9}. (C) Comparison between the obtained results for the $TCID_{50}$ based on the three methods that have been used for this experiment (qualitative vs quantitative vs MTT assay methods).

Optimization of the $TCID_{50}$ protocol

To optimize the $TCID_{50}$ protocol, various experiments were performed, testing different number of cells, different FBS concentrations that the cell media was supplemented with, but also viral concentrations and incubation times.

At first, different number of MRC-5 cells were seeded: 2500, 10.000 and 20.000 cells/well with a 3h incubation prior the infection with a concentration of 10^{-1} of HCoV-229E. In the plates containing 2500 and 10.000 cells, the cell media (DMEM) was supplemented with 2% FBS, whereas in the 20.000 cells plate, the FBS concentration in the cell media was 5%. The OD readings obtained for 10.000 cells seeded indicated a remarkable difference (0.33), since the mean OD obtained for infected cells was 0.26, whereas for the not-infected cells, the OD mean was 0.59. When 2500 cells/well were seeded, the OD values obtained for the infected cells (mean = 0.95) were slightly higher than the ODs for the not-infected cells (mean = 0.88). When the OD values from 20.000 cells/well experiment were obtained, a similar value was obtained for both the infected (mean = 0.67) and not-infected cells (0.76) (Figure 2A).

In the following experiments a number of 10.000 cells was tested once again, but with a 24h incubation prior infection. Here, the cells were seeded in DMEM 10% FBS. The OD values were rather similar, the difference in the OD mean values being an average of 0.11 (Figure 2B).

Lastly, 2500 and 10.000 cells were tested again, this time the cells being incubated until they reached confluency and then infected with HCoV-229E. Once again, for the 10.000 cells experiments, the resulted OD readings revealed a difference of 0.37 in the cell viability between infected and not infected cells (Figure 2C).

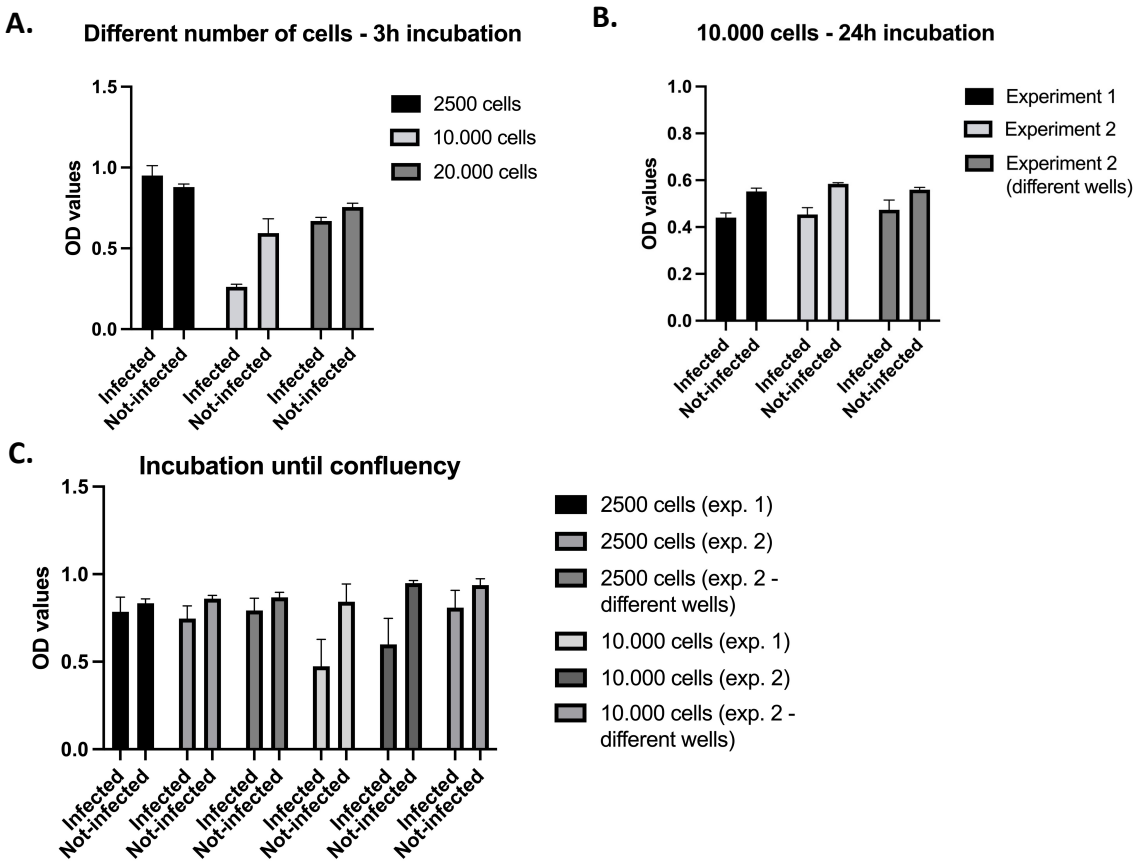

Figure 2. Bar charts representing the cell viability of MRC-5 cells infected with a concentration of 10^{-1} of HCoV-229E, the bars representing mean values ± SD. (A) OD readings for different number of cells comparing the viability of infected vs not-infected cells. 2500 and 10.000 cells were seeded in DMEM 2% FBS, whereas 20.000 cells in DMEM 5% FBS. The cells were infected after 3h of incubation. (B) OD values obtained after two independent experiments where 10.000 cells were seeded in DMEM 10% FBS with 24h incubation prior the infection. (C) OD readings resulted from two independent experiments where 2500, 10.000, 20.000 cells were seeded in DMEM 10% FBS and incubated until reached confluency before the infection.

It is relevant to mention that other conditions were also tested, such as seeding the three number of cells (2500, 10.000, 20.000) using cell media supplemented with multiple FBS concentration, such as 2%, 5%, 8%, 10%. The cells were infected with different concentrations of HCoV-229E, that ranged from 10^{-1} to 10^{-9}.
Furthermore, OD readings were collected after trying multiple staining methods, such as MTT assay, neutral red uptake assay or crystal violet staining method. The neutral red uptake assay and crystal violet staining method were unsuccessful, due to the fact that the OD values generated by the plate reader indicated no remarkable difference between the cell viability of the infected cells versus the not infected cells. Therefore, these results were not in accordance with what has been noticed at the microscope.
All conditions were evaluated in at least two independent experiments and based on these, it was decided that the best way to proceed with the project was to seed 10.000 cells/well in DMEM 10% FBS, with a 3h incubation time prior infection with HCoV-229E.

HCoV-229E CPE-based antiviral assay
Several compounds known to be active against the main protease (M^{pro}) of different CoV were tested in the optimized HCoV-229E CPE-based antiviral assay. The antiviral effect of compounds **19**, **nirmatrelvir (PF-07321332)** and **GC-376** was investigated in MRC-5 cells infected with HCoV-229E. Compound **19** demonstrated its ability to inhibit the CPE

development by HCoV-229E infection, in a dose-dependent manner. Thereby, the EC_{50} value for compound **19** was 0.22 μM (Figure 3A) and no cytotoxicity was noticed for the highest concentration tested. The cytotoxicity was evaluated based on the visualization of cells under the microscope and comparing their appearance to the control cells (uninfected-untreated). Compounds **nirmatrelvir** and **GC-376** were used as reference compound to confirm that the designed assay worked. These two compounds inhibited the CPE development with an EC_{50} value of 1.24 μM for **GC-376** (Figure 3B), whereas for compound **nirmatrelvir** the EC_{50} was 0.72 μM (Figure 3C). No cytotoxicity was observed when these compounds were tested. From the novel compounds tested at 20 μM and 10 μM (**MP11**, **MP16**, **MP17**, **MP18**, **MP19** and **OVE-002 (12-1)**), only **MP17** and **MP19** indicated an ability to inhibit the antiviral activity of HCoV-229E in MRC-5 cells (Figure 3D).

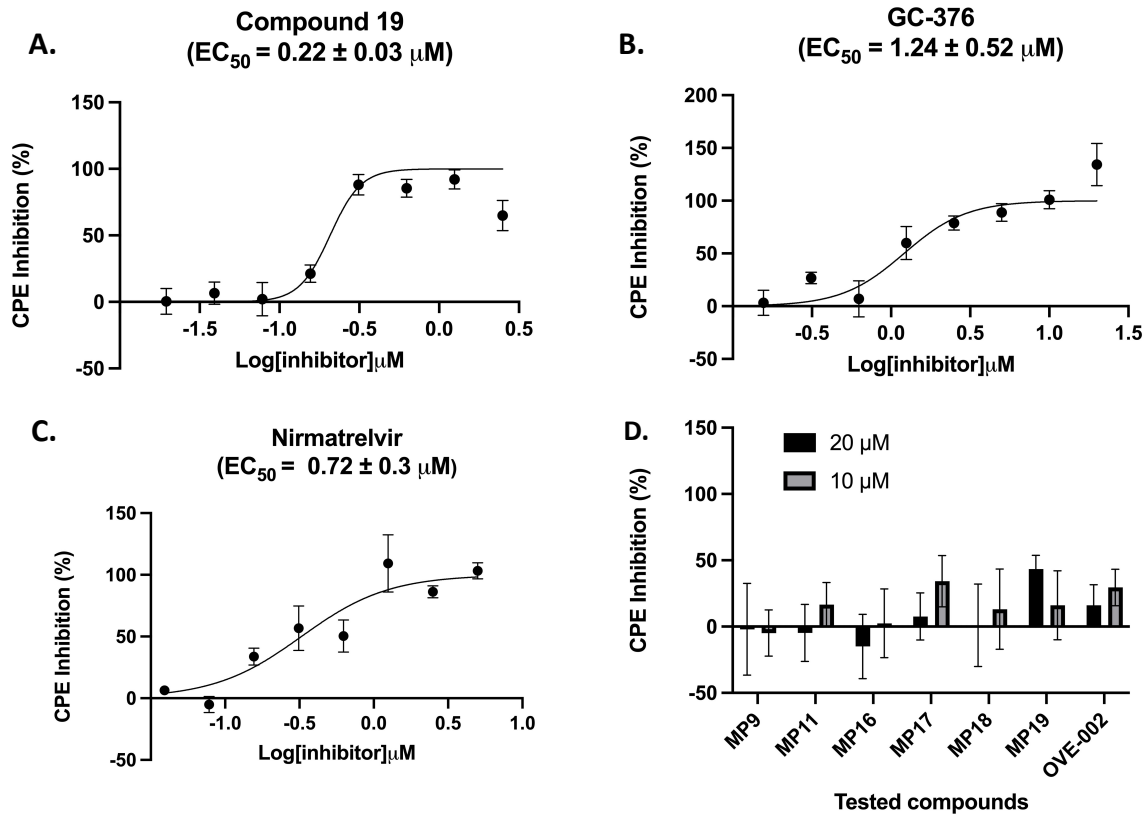

Figure 3. Inhibitory concentrations of the selected compounds on CPE caused by HCoV-229E infection in MRC-5 cells. The CPE-based assay was performed by using 10.000 cells incubated for 3h, followed by the infection with a viral concentration of 10^{-1}. After 5 days pi, absorbance was read based on a MTT assay. The EC_{50} was expressed as mean ± SEM out of triplicates from two independent experiments.

Discussion

One of the important virus-based targets that represent an interest when attempting to develop novel drugs against Coronavirus is its main protease (M^{pro}). Several compounds have been previously tested against M^{pro} of multiple CoVs, such as SARS, MERS, or SARS-CoV-2. Therefore, this present project aimed to investigate their antiviral potency also against the M^{pro} of a different CoV, namely the human coronavirus 229E (HCoV-229E). To be able to achieve this goal, the first step taken consisted in the identification and possible optimization of a suitable protocol.

Among the reasons behind focusing on the main protease of CoV is their key role in the viral replication (Zhang et al., 2020). Apart from this, it was mentioned in the literature that the M^{pro} is also a region of the genome with an elevated level of conservation between the different coronaviruses (Matthews et al., 2020). To have a better understanding of this level of conservation, a multiple sequence alignment was run for the M^{pro} sequences of SARS, MERS, SARS-CoV-2 and HCoV-229E. Indeed, there is a degree of similarity between these sequences and for example, the M^{pro} of HCoV-229E is most similar to MERS protease (49.34%), but it also shares approximately 40% with the M^{pro} of SARS and SARS-CoV-2 sequences (Table 1). In addition to this, it is different from the human cysteine proteases since in humans no protease has a similar cleavage specificity (Qiao et al., 2021). This has led to the conclusion that a drug against the M^{pro} of Coronaviruses will not have repercussions following the administration to humans (Hu et al., 2022). All these reasons strengthen the choice of targeting this protease, and that is why the main protease is an optimistic target for the antivirals against Coronaviruses.

Starting from previous published literature, it was decided that in order to set up a cell-based assay appropriate for the HCoV-229E infection, cells that are able to develop CPE caused by this virus should be chosen. Thereby, the MRC-5 cells, which are human fibroblast lung cells were selected. Previous studies have demonstrated that these cells could be utilized for the HCoV-229E propagation and compounds testing (Hu et al., 2022), but also for the viral titration (Funk et al., 2012; Bracci et al., 2020).
In this present project, when the $TCID_{50}$ experiment was performed, three methods were required for data analysis. As it conducted usually, the first method, namely method 1 – qualitative, was based on the visualization of the cells under the microscope. When comparing the healthy cells with cells that presented CPE, the $TCID_{50}$ value was determined to be 10^{-5}. To be more accurate, other methods were taken into consideration. The second method, called method 2 – quantitative, assessed the cell viability. Based on the obtained OD readings, this method indicated that the $TCID_{50}$ value was 10^{-3}. As it was previously mentioned, due to the difference in the $TCID_{50}$ values (Figure 1A), a third method was required to verify which method was more precise. Thus, the MTT cell viability assay method, evaluating which viral concentration caused 50% CPE, was utilized. According to the analysis effectuated in the GraphPad software, the $TCID_{50}$ value was $10^{-2.9}$ (Figure 2B). To highlight the similarities and differences in the obtained $TCID_{50}$ values, all data was plotted in a graph (Figure 3C). This comparison demonstrated that method 2 – quantitative is more accurate, the result being confirmed with the MTT assay method. When it comes to method 1 – qualitative, the difference was rather high, which could mean that the interpretation of the infected wells only based on the CPE development might be misleading. This could be explained by the fact that even if some cells did not show much CPE, the infection still affected the cells, generating such a decreased cell viability demonstrated through the absorbance (OD) readings.
It is relevant to mention that these experiments were repeated and differences in the results appeared. This could have happened because of the low titer of the viral stock (1.3×10^3 PFU/ml), therefore a viral stock with a higher titer would be suggested for following experiments. Additionally, these differences required an optimization of the $TCID_{50}$ protocol. To do so, various conditions were tested, such as different number of cells, different FBS concentrations in the used cell media or incubation times prior the infection. In accordance with the protocol published by Hu et al. (2022), MRC-5 cells at a density of 2500 cells/well were seeded. A choice of also seeding 10.000 cells was made, and lastly, 20.000 cells (Owen et al., 2021). The cells were incubated for 3h before infection with HCoV-229E, followed by a secondary incubation of 5 days to allow CPE formation. Then, OD readings were taken for the cell viability. Ideally, the OD readings for the not-infected cells should have been as close as possible to 1, as this is generally obtained when cells are confluent or close to confluency, with

an important difference compared to the OD readings for infected cells. However, the results obtained in this project indicated that no major difference was recorded between the OD readings when 2500 and 20.000 cells were seeded. In the case of 2500 cells, almost no difference in the OD values was read and when visualizing the wells under the microscope, almost no CPE was noticed. When it comes to the 20.000 cells experiments, the not infected cells were not healthy, with a tendency towards lysing. This could be explained through the fact that the cells overgrew. The obtained result can be compared to a study carried out by Owen et al. (2021), where a number of 20.000 cells were used for HCoV-229E antiviral assay. Different outcomes were recorded, but this can be justified by the fact that a 24h incubation before the infection took place, and the infected cells were incubated for 3 days, which was slightly dissimilar to what has been performed in this project. According to the published article, 3 days pi should have been enough, but here the cells did not show any sign of CPE development. However, good results were obtained when 10.000 cells were seeded, since the difference between the OD values of infected cells versus the OD values of the not infected cells was as expected (Figure 2A).
Since a great difference was seen between the OD values obtained when seeding 10.000 cells, the optimization of the protocol continued with seeding again 10.000 cells/well, but this time being infected succeeding a 24h incubation. The results gained from two independent experiments (two sets of wells were analyzed for experiment 2) were not satisfactory (Figure 2B), since OD readings were not as expected due to insufficient difference between the OD values and thus, the decision of not continuing with this protocol has been taken.
Lastly, other experiments were conducted by seeding 2500 and 10.000 MRC-5 cells, followed by incubation until confluency before infecting them with HCoV-229E. Differences in OD values were achieved once more when testing 10.000 cells (Figure 2C). However, this result was not consistent among the experiments, as infected cells had variable OD values. Moreover, even if a desired OD difference was obtained, the experiment itself became time-consuming since it lasted approximately 7-8 days to obtain results.

When testing different staining methods to assess cell viability, the one that stood up was represented by the MTT assay. The neutral red uptake assay and the crystal violet staining method were not adequate and when the absorbance was read the obtained values were unsatisfactory. A reason behind this could be that the chosen staining methods have a different mechanism when it comes to how the cell viability is measured for each procedure. To be more precise, the MTT assay assesses the cell viability based on the ability of living cells to lead to the formation of formazan crystals from MTT through an active mitochondria (van Merloo et al., 2011). The neutral red uptake assay depicts alive cells with the capacity of binding and incorporation of neutral red dye (Ates et al., 2017). In this study, a probable cause for the unsuccessful assay can be explained through the fact that even when cells developed CPE, some of them were still able to take-up the neutral red, leading to insufficient difference in obtained OD values. The crystal violet staining method is based on staining the attached cells, considering that dead cells have been detached and washed away from the wells (Feoktistova et al., 2016). During present experiments, the MRC-5 cells were not all detaching when the CPE was formed, thus the majority of cells were stained justifying the undesired OD values.
As a consequence of all these experiments, also considering the efficacy and effectivity, the $TCID_{50}$, i.e., the CPE-based assay that was set up, was based on seeding 10.000 MRC-5 cells in DMEM 10% FBS, followed by a 3h incubation prior infection with HCoV-229E (10^{-1}). To allow CPE formation, the cells were subsequently incubated for 5 days, and the cell viability was detected performing the MTT assay.

Different compounds targeting the M^{pro} of Coronaviruses were tested against the infection with HCoV-229E. The first compound tested was compound **19**, which belongs to noncovalent and nonpeptidomimetic inhibitors of M^{pro} (Kitamura et al., 2022; Luttens et al., 2022). It is a compound that have been previously demonstrated to be active against SARS (EC_{50} = 0.39 μM), MERS (EC_{50} = 0.20 μM) and SARS-CoV-2 (EC_{50} = 0.11 μM) (Luttens et al., 2022). As expected, this compound demonstrated its efficacy also against the M^{pro} of HCoV-229E, with an EC_{50} value of 0.22 μM (Figure 3A). Therefore, this study strengthens the previous findings, and it demonstrates once more the broad-spectrum anti-coronaviral effect of compound **19**.
To validate the success of the designed CPE-based assay, **GC-376** was used as reference compound due to its known antiviral potency mainly against the M^{pro} of a CoV infecting cats, namely the Feline Infectious Peritonitis (FIV) CoV (Pedersen et al., 2018). This compound has also been tested against a large panel of CoVs, including both HCoV-229E (EC_{50} = 0.12 μM) (Hu et al., 2021) and SARS-CoV-2 (EC_{50} = 3.37 μM) (Ma et al., 2020). In the protocol published by Hu et al. (2022), **GC-376** had an EC_{50} = 0.078 μM. The values seem to vary, and this might be explained by the viral load. However, the result from the present study was in accordance with the published literature. Thus, **GC-376** is an effective compound against HCoV-229E (Figure 3B), but it also has a broad-spectrum efficacy.
Nirmatrelvir (PF-07321132), an already approved drug against SARS-CoV-2, was also used as a reference compound. The antiviral potency of this compound against HCoV-229E re-confirmed the success of the optimized protocol, obtaining an EC_{50} = 0.72 μM (Figure 3C). Furthermore, the obtained value was compared to the previous obtained EC_{50} values in the study performed by Owen et al. (2021), where the EC_{90} = 0.62 μM.
Ultimately, the potency of the novel compounds (**MP11**, **MP16**, **MP17**, **MP18**, **MP19** and **OVE-002 (12-1)**) was investigated. The results indicated that only compounds **MP17** and **MP19** have an antiviral effect against the HCoV-229E infection (Figure 3D).

As a conclusion of what has been presented, the aim of this study, which was to set up a HCoV-229E CPE-based antiviral assay, was achieved. The success was confirmed by using two reference compounds (**GC-376** and **Nirmatrelvir**), thus the CPE caused by HCoV-229E was inhibited. Additionally, compound **19**, which was known to be active against several CoVs (SARS, MERS, SARS-CoV-2), was demonstrated to be a broad-spectrum inhibitor of CoVs based on its ability to also inhibit the CPE produced by HCoV-229E.
Another interesting outcome was represented by the antiviral activity of the novel compounds **MP17** and **MP19**. These compounds showed a great potential to inhibit CPE development caused by HCoV-229E. Since only two concentrations were tested for these compounds, a future perspective would be represented by the further investigation of the anti-coronaviral activity of these compounds. Thus, multiple concentrations should be tested in CPE-based assays which would lead to the finding of an EC_{50} value.

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