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In Vitro Assessment of Novel Compounds as Potential Pan-Coronavirus Therapeutics in SARS-CoV-2 and *In Vitro* Assessment of a Pan-Flavivirus Compound in Zika virus

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Abstract

Through the SARS-CoV-2 pandemic, it has become clear that the development of antivirals is essential for the health and wellbeing of the population. In this study, novel active site protease inhibitors against SARS-CoV-2 were tested for their inhibitory activity against the viral 3-Chymotrypsin like protease through the means of FRET based enzymatic assays. Additionally, Compound 104 targeting the NS2B-NS3 protease was tested against Zika virus through yield reduction assays as a means to assess whether these assays are suitable for the assessment of peptide hybrid compounds in Zika virus.

Novel compounds against SARS-CoV-2 were screened and five of the selected six active compounds were found to inhibit the viral protease at a half-maximal inhibitory concentration (IC_{50}) of below 0.075 µM.

In Zika virus, the yield reduction assay was assessed and it was found that under the conditions tested, this assay is not suitable for the assessment of peptide hybrid compounds in Zika virus.

The active novel compounds against SARS-CoV-2 should be taken for further assessment in cell based assays as the next step of development. Compound 104 should be assessed under different experimental conditions to identify whether different conditions can make this assay suitable for the intended use.

Key Words

Human coronavirus, active protease inhibitors, drug discovery, 3CLpro, Zika virus, flavivirus inhibitor, NS2B-NS3

Popular Scientific Summary

Potential New Drugs for Treatment of SARS-CoV-2 and Zika Virus Infection

Viral diseases have been known for a long time, and treatment for them is essential for the health and wellbeing of many people. One of the most prominent viral diseases at this point in time is coronavirus disease-19 (COVID-19), the current pandemic caused by the coronavirus SARS-CoV-2. While there are vaccines available now, and the first approved treatment for acute COVID-19 is on the market, there is still a need for more antiviral drugs, due to the high number of people who are getting infected and the possibility of the virus developing resistance against the current drugs. One way to treat the disease is to stop the virus from replicating, which can be targeted in multiple steps of the virus life cycle. In this study, novel compounds were tested for their ability to inhibit the making of new building blocks to create more virus.

Compounds underwent multiple rounds of testing to determine which of these were active and six compounds with promising results were identified for further drug testing. In case of a good drug candidate being found, there is a possibility of this candidate being useful to treat more than just SARS-CoV-2 infection. This is because the coronaviruses that are able to infect humans share a high similarity in their mechanisms, and thus a drug targeting one of those mechanisms could potentially inhibit more than one human coronavirus.

A second common viral disease is caused by Zika virus. Zika virus is spread through the bites of some species of mosquitoes. These mosquitoes are spreading further across the globe as global warming and other factors work together, and so the high number of cases is thought to increase even further. Infection oftentimes presents as fever, pain and muscle aches. However, in the case of a pregnant person becoming infected, this disease can cause birth defects in infants, such as microcephaly, which is a condition in which an infant's head is too small, or stops growing after birth. Thus, it is important to be able to treat this infection, and currently, no treatment is available. Here, one compound that targets the same step as in the coronavirus antivirals – the making of new virus building blocks - was tested against Zika virus, to determine if the way of testing is appropriate for drug assessment with this type of test. The results gathered in this study do not support the theory that it is possible to use the experiments used here to assess a specific type of drug.

Introduction

Due to the current pandemic, the SARS-CoV-2 coronavirus is known all across the globe. However, coronaviruses have been discovered in relation to human diseases already in the 1960s (1), where they have been isolated as causes of the common cold. Taxonomically, the coronaviruses (CoV) are classified into the order *Nidovirales* and the family *Coronaviridae*. Further classification divides the *Coronaviridae* into the sub-families *Orthocoronavirinae* and *Letovirinae* (2). On the basis of phylogeny, these subfamilies are further divided into four genera, the *alpha*, *beta*, *gamma* and *delta* CoV (2).

There are seven known human coronaviruses (1,2), *Table 1*, which are part of the *alpha* and *beta* genera. Within the highly pathogenic coronaviruses, SARS-CoV and MERS-CoV belong to the subgroups 2b and 2c of the *beta* coronavirus genus, while the recently discovered SARS-CoV-2 has been shown to be a distinctly different coronavirus (3). Even so, SARS-CoV-2 shares 86.8% sequence identity with SARS-CoV and 81.2% sequence identity with MERS-CoV (4). The highly pathogenic coronaviruses SARS-CoV and MERS-CoV are thought to have originated in bats (5), the same as the other known human coronaviruses (6). For the newest human coronavirus, SARS-CoV-2, current theories predict a zoonotic origin as well (7,8).

Table 1 Classification of human coronaviruses.

Genus	Species	Species Abbreviation
α-CoV	Human coronavirus 229E	HCoV-229E
	Human coronavirus NL63	HCoV-NL63
ß-CoV	Human coronavirus OC43	HCoV-OC43
	Human coronavirus HKU1	HCoV-HKU1
	Severe Acute Respiratory Syndrome coronavirus	SARS-CoV
	Middle East respiratory syndrome coronavirus	MERS-CoV
	Severe Acute Respiratory Syndrome coronavirus 2	SARS-CoV-2

(9)

Human coronaviruses are the source of many different diseases, often causing gastrointestinal tract symptoms and respiratory symptoms (1,10), which vary in severity from self-limiting to severe disease manifestations such as pneumonia and acute respiratory distress syndrome. (1,11). The human coronaviruses HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-KHU1 have been identified as causes of the common cold (11). The highly pathogenic human coronaviruses, SARS-CoV, MERS-CoV and SARS-CoV-2, which have emerged in 2002 (12), 2012 (13) and 2019 (14) respectively, can cause fatal diseases (15). To combat the mortality caused by the highly pathogenic human coronaviruses, developing effective treatment is

prudent, especially for SARS-CoV-2, which is much more common than cases of MERS-CoV. Drug development against SARS-CoV is not as pressing, as the last case of this has been recorded in 2004 (16). Depending on the country, SARS-CoV-2 has a case fatality of 0.0%-18.1% (17), while MERS-CoV and SARS-CoV have a mortality of approximately 30%-40% and 10% respectively (15). Transmission of these viruses occurs mainly through respiratory droplets which are spread from person to person (18). This caused rapid spread of the virus, and it had been confirmed in almost every country within six months of the beginning of the pandemic (19).

During this SARS-CoV-2 pandemic, more than 517 million people have been infected, and over 6.2 million deaths have been recorded (20). MERS-CoV and SARS-CoV, on the other hand, have not spread quite as far. MERS-CoV, which was discovered in Saudi Arabia (13), occurs mainly in the Middle East when cases arise (21). SARS-CoV spread to a total of 37 countries during the outbreak in 2002/2003, but no new cases have occurred since 2004 (16). The lowly pathogenic human coronaviruses have been shown to also be spread globally, with more cases occurring in winter, and children being more commonly affected than adults (22).

Following the SARS-CoV outbreak in 2002/2003 and the MERS-CoV outbreak in 2012, SARS-CoV-2 has caused the third outbreak of coronavirus origin within the 21st century (23). While there are now vaccines available against SARS-CoV-2, current data shows that even with vaccine protection, patients are still at risk to become infected (24). Additionally, since new variants are continuously evolving, such as the most recent Omicron variant, there is also a chance of re-infection occurring, even with vaccination and it is thought that protection by the vaccine will decline over time (25).

As the majority of the human coronaviruses cause only mild disease, treatment options mainly consist of supportive care in the form of resting, in-take of fluids and analgesia (10). However, in the cases of the highly pathogenic coronaviruses, this treatment is often not enough. Commonly, broad spectrum antibiotics, antivirals, IFNs or antifungals are prescribed to reduce the risk of co-infection with opportunistic pathogens (26), but the virus itself is rarely targeted.

Previous medications commonly prescribed to directly treat SARS-CoV-2 infection, such as ribavirin and corticosteroids, have been ineffective (27). However, corticosteroids can reduce the severity of coronavirus-19 disease (COVID-19) by reducing the induced inflammatory response (28). Remdesivir, a nucleoside analogue, also has limited use, as it is removed by the virus´ RNA dependent RNA polymerase (RdRP) proofreading ability (29,30). Even so, it is approved for use in adults and certain paediatric patients (31).

Acute infection is now targeted by various drug development programmes, with many drug candidates undergoing clinical trials (32). Most are immunomodulators, and only a minority are antivirals. The food and drug administration has granted emergency use authorisation of three monoclonal antibody therapies to treat SARS-CoV-2 infection (33). Of these three, only sotrovimab has been shown to be effective against the currently, as of the 3rd of March 2022 (34), predominant Omicron variant (33,35), and has therefore been conditionally recommended by the World Health Organisation (WHO) (35).

Structure based drug design makes use of viral proteins to inhibit various parts of the virus life cycle. Thus, it is important to know how coronaviruses are built. Structurally, these are enveloped viruses with a positive sense, single stranded RNA genome, ranging from 26 kb to 32 kb in size, making them one of the largest RNA viruses to date (1). Within the genome, the

open reading frames (ORF) 1a/b at the 5´ terminal encode 16 non-structural proteins (nsp1-16). The structural proteins, namely spike, envelope, membrane and nucleocapsid proteins are encoded by a second ORF towards the 3´ terminal (1). Three proteins that have been considered for antiviral drug design in SARS-CoV-2 are the Papain-like protease, the main protease and the RNA dependent RNA polymerase (36). The cysteine proteases Papain-like protease (PLpro) and the 3-chymotrypsin-like main protease (3CLpro or Mpro) are encoded on ORF 1a (37). They are essential enzymes for viral replication, cleaving the polyproteins pp1a and pp1ab which are encoded by ORF 1a into the distinct non-structural proteins (38–42). While PLpro cleaves the first three cleavage sites, the 3CLpro, *Figure 1-A*, is responsible for the cleavage of the remaining sites (37), *Figure 1-B.*

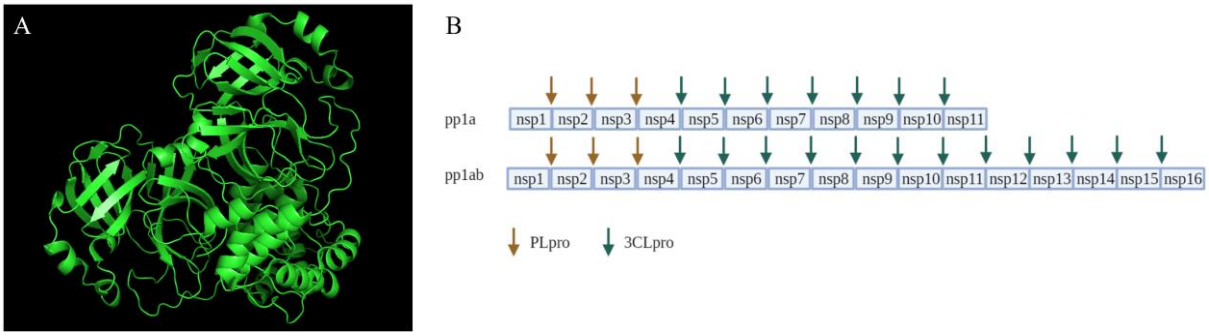


Figure 1. 1-A: Structure of the SARS-CoV-2 3-Chymotrypsin like protease in its homodimeric form. Figure was created with Pymol and the Protein Data Bank file 7P35. 1-B: Schematic representation of the cleavage of the viral pp1a and pp1ab polyproteins by the two human coronavirus proteases, the Papain-like protease (PLpro) and the 3-Chymotrypsin like protease (3CLpro). Cleavage sites are indicated by arrows, brown for cleavage sites of the PLpro, and green for cleavage sited of the 3CLpro. The order of the non-structural proteins (nsp) within the viral polyproteins is indicated by individual light blue blocks. Adapted from (23), created with BioRender.

In its active state, the 3CLpro is in a homodimeric conformation, which it assumes in the presence of an appropriate substrate (37). Each of the monomers is known to be composed of three structural domains, of which domains I and II form a chymotrypsin-like structure (43). Furthermore, this enzyme has a catalytic dyad of cysteine (C145) and histidine (H41) in its active centre (44–47). Instead of a third catalytic residue, a water molecule is present in the active site (48–50).

Proteases are a good drug target due to their essential function during the virus life cycle. Prior research into protease inhibitor development during the SARS-CoV outbreak in 2002/2003 has provided a starting point for drug testing and development for targeting the SARS-CoV-2 3CLpro (51–53). Inhibitors of various compositions have been designed, and strongest inhibition is currently achieved by ketone based covalent inhibitors (54). Recently, the oral 3CLpro inhibitor Paxlovid (nirmatrelvir and ritonavir) by Pfizer has been approved for use in the European Union (55), as well as in other parts of the world. While this is a success for the treatment of COVID-19, it is still of interest to have access to multiple drugs developed for the treatment of SARS-CoV-2 due to the large number of patients suffering and the possible development of drug resistance by the virus.

Similar to the coronaviruses, Zika virus, a virus of the *flavivirus* genus within the *Flaviviridae* family, is also a positive sense single stranded RNA virus (56) with a size of approximately 10.8 kb. Its genome encodes one polyprotein that encodes three structural proteins and seven non-structural proteins (57). The structural proteins are the capsid (C), the pre-membrane (prM) and the envelope (E) protein (58). The non-structural proteins – namely NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 – are essential for viral function, including genome replication and polyprotein processing (57).

The primary transmission pathway of Zika virus to humans is through the bite of an infected *Aedes* mosquito, most commonly *Aedes aegypti* (59). Oftentimes, the disease caused by Zika virus is mild, including a fever, rash, conjunctivitis, muscle and joint pain, malaise or headaches for a period between two and seven days (60). Upon infection during pregnancy, however, Zika virus can cause congenital Zika syndrome, which can result in microcephaly or other congenital malformations in infants (60). Transmission of Zika virus has been reported in 86 countries so far (60), with current or previous areas with transmission being in North and South America, countries in Sub-Saharan Africa and Asia. However, areas where there are established vector mosquitoes without recorded cases so far spread most of Africa and Asia, as well as Australia (60).

Initially identified in a Rhesus monkey in 1947 from the Zika Forest of Uganda, Zika virus was then identified in humans seven years later (61). Yearly, Zika virus infections are reported, and between the years 2016 and 2019, a total of 84,276 cases were registered (61). In general, outbreaks of flavivirus infections occur worldwide both in humans and in animals (62) and the geographical spread of flaviviruses, such as the West Nile and Zika virus, was rapid (62).

At this point, there is no specific treatment available for Zika virus infection (63,64), and in case of disease developing it is recommended to take medication to reduce fever and pain. Steps in the virus life cycle that are targeted with drug development include viral attachment and entry, the protease, and the RdRP, among others (62,64–66). The flavivirus protease, *Figure 2-A*, the NS2B-NS3 complex, where NS2B is a cofactor and NS3 includes the protease domain (67), is highly conserved among the flaviviruses and is an essential enzyme for virus replication (68,69), making it an attractive target for the development of pan-flavivirus treatment. Its role is to cleave part of the flavivirus polyprotein that is produced during virus replication (70), whereas the remaining cleavage is done by host cellular proteases, *Figure 2-B*.

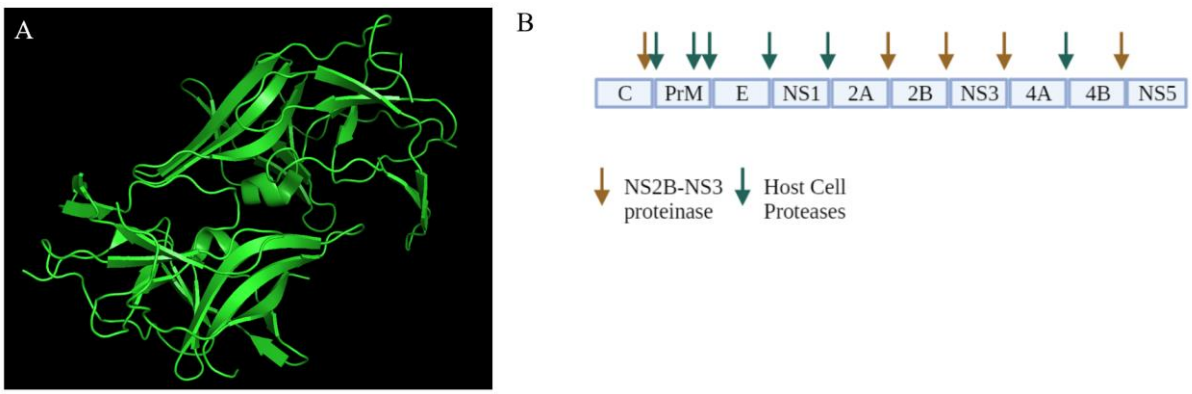


Figure 2. 2-A: Structure of the ZIKV NS2B-NS3 protease complex, created with PyMol with the use fo the Protein Data Bank file 5GXJ. 2-B: Schematic representaion of the protease cleavage of the Zika virus polyprotein by

NS2B-NS3 protease and host cell proteases, where brown arrows indicate cleavage by he viral protease, and green arrows indicate cleavage by host cell proteases. The order of the non-structural proteins (nsp), as well as the capsid (C), pre-membrane (PrM) and envelope (E) protein within the viral polyprotein is represented by light blue blocks. Adapted from (67), created with Biorender.

Problems with the development of active site protease inhibitors have arisen due to the flat and featureless active site of this enzyme, as well as a negative charge due to aspartate residues and many of the inhibitors developed have only shown effectivity in biochemical assays, but low effectivity in cell based assays and *in vivo* (68). Additionally, the active site of the NS2B-NS3 complex is structurally similar to that of serine proteases found in the host, potentially causing protease inhibitors to have adverse effects on host cells (70).

Given the relevance of the need for new antiviral drugs against these viruses, this study investigated novel compounds targeting the protease active site through FRET based enzymatic assays for their inhibitory potential against the SARS-CoV-2 3CLpro *in vitro*. Furthermore, a potential pan-flavivirus compound targeting the flavivirus active site was assessed for its inhibitory effect against Zika virus through yield reduction assays.

Aim

1. The first aim of this study is to investigate the coronavirus inhibitory activity of novel SARS-CoV-2 3CLpro active site inhibitors in SARS-CoV-2 3CLpro through determination of the compounds´ half maximal inhibitory concentration (IC_{50}).

2. The second aim of this study is to assess the yield reduction assay as a method to test peptide hybrid compounds with the aid of a peptide hybrid, Compound 104.

Materials and Methods

1. Cells, Virus and Medium

VERO-E6 cells (African green monkey kidney cells) were cultured with Dulbecco´s Modified Eagle Medium (DMEM, Gibco, Cat #11965-092) supplemented with 10% foetal calf serum (FCS, Gibco, Cat #100500-064) and 1% Penicillin-Streptomycin (Gibco, Cat #15140-122), from here on referred to as DMEM-10. Cells were cultured in an incubator at 37°C and 5% CO_2. HUH7 cells (human hepatoma cells) were cultured in DMEM-10 under the same incubation conditions as the VERO E6 cells.

Zika virus (strain MR766, kindly provided by Olli Vapalathi) stocks were made by infecting a 25 cm^2 tissue culture flask of VERO E6 cells with Zika virus at a multiplicity of infection (MOI) of 0.1 from a known virus stock of 6.35×10^5 that was assessed by plaque assays. Zika virus inoculum was prepared in DMEM supplemented with 2% FCS and 1% Penicillin-Streptomycin, from now on referred to as DMEM-2. Cells were washed with PBS, and then 2.5

ml of the virus inoculum was added. Flasks were rocked in a cross-like motion every 15 minutes for one hour for even distribution of the virus. Then, 2 ml of DMEM-10 were added and flasks were incubated at 37°C and 5% CO_2. At 48 hours, 72 hours and 96 hours post infection (pI), 300 µl of the supernatant were taken and frozen at -80°C for determination of virus quantity at the individual time points. Removed volume was replaced with 300 µl of DMEM-10. Virus concentration was then assessed through plaque assays.

2. Virus Titration by Plaque Assay

Very E6 cells were seeded at an amount of 6.5 x 10^5 cells/well for 6-well tissue plates and at an amount of 2.2 x 10^5 cells/well for 24-well tissue plates in DMEM-10 at a total volume of 2 ml and 1 ml respectively. Zika virus was diluted in a 1:10 serial dilution from stock to 10^{-6} in DMEM-2. After 24 hours, the media was removed from the wells, and the cells were infected with 100 µl of the diluted virus. DMEM-2 was used for negative controls. Infected cells were incubated for 1 hour at 37°C and 5% CO_2 and plates were rocked in a cross-like pattern every 10 minutes to prevent drying and damaging of the cell´s monolayer, as well as for even virus distribution. After incubation, an overlay consisting of a 1:1 ratio of 1% noble agar (Sigma Aldrich, Cat #A5431-250G) and 2 x Modified Eagle Medium (MEM, Gibco, Cat #11935-046) which was kept liquid in a 45°C water bath, was added to the wells. In 6-well tissue plates, 2 ml overlay was added, whereas in 24-well tissue plates, 500 µl of overlay were added. Afterwards, the plates were incubated at 37°C and 5% CO_2 for 4 days. Then, cells were stained by adding 3% neutral red solution (Sigma Aldrich, Cat # N2889-100ML) in PBS to each well, 2 ml in 6-well tissue plates, and 500 µl in 24-well tissue plates. After incubation of 2-4 hours, the remaining neutral red solution was removed, plaques were counted and relative PFU (plaque forming units) was calculated ($\frac{Number\ of\ Plaques}{volume\ of\ inoculum\ x\ dilution\ factor}$).

3. MTT Assay

96-well plates were seeded with 10^4 HUH-7 cells per well and incubated for 2 hours at 37°C, 5% CO_2 for cells to attach. Then, cells were treated with compounds, at two-fold concentration gradients between 50 µM and 1.5625 µM for Compound 104, and at 200 µM and 20 µM for compound AEBSF. Plates were incubated for 24 hours at 37°C, 5% CO_2 and MTT assays were conducted as described before (71).

4. Yield Reduction Assay

A 96-well plate was seeded with 10^4 HUH-7 cells per well in a DMEM-2 total volume of 100 µl. Plates were incubated for two hours at 37°C, 5% CO_2 to allow cells to adhere to the wells. Medium was then removed, and replaced with 50 µl fresh DMEM-2. Cells were treated with Compound 104 at concentrations ranging from 50 to 1.56 µM as follows. First, Compound 104 was two-fold serially diluted in DMEM-2 to x4 final wanted concentration (200 µM to 6.26 µM), then cells were treated by addition of 25 µl of the different compounds´ dilutions. Cells were then infected with 25 µl of viral inoculum to an MOI of 1. This final step brought the final volume to 100 µl effectively diluting the compounds 1:4 (25 µl of compound into a final volume

of 100 µl) to the final wanted working concentration. Plates were incubated at 37°C, 5% CO_2 and supernatant was collected 24 hours later. Supernatant was frozen at -80°C until use.

Reference compound AEBSF was tested in the same setup at concentrations of 200 µM and 20 µM.

Virus quantity within the supernatants was titrated through plaque assays and expressed as PFU/ml, as described above.

5. SARS-CoV-2 3CLpro Activity Assay

Enzymatic assays were done manually as described before (72) in black 96-well assay plates. Compounds´ stock (10mM) were serially diluted, first in DMSO, then in water, to x10 the final wanted working concentration at a total volume of 1 ml. By adding 10 µl of the compound dilutions per well, the final volume was brought to 100 µl (10 µl substrate, 10 µl 3CLpro, 10 µl compound dilution and 70 µl buffer), and compounds were effectively diluted 1:10.). Buffer used in this assay was 20 mM HEPES (Gibco, Cat#15630-080) with 0.01% TRITON-X-100 at a pH of 7.5. The SARS-CoV-2 3CLpro recombinant enzyme was produced in collaboration with the Protein Science Facility, Karolinska Institute, Sweden (72). Before addition of the internally quenched substrate (DABCYL-Lys-Thr-Ser-Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg-Lys-Met-Glu-EDANS), which was produced by Bachem Holding AG (Switzerland), the protease was allowed to incubate with the compound for 10 minutes by letting the plate sit at room temperature for 10 minutes. For the initial screening, compounds were tested at final concentrations of 1 µM and 0.1 µM to determine initial velocities. Fluorescence increase was measured at 355 nm excitation and 538 nm emission every 60 seconds for 40 minutes at 37°C with the Tecan infinite® 200 plate reader. After initial assessment, active compounds were tested at concentrations ranging from 2 µM to 2 x 10^{-4} µM for determination of the IC_{50} (concentration at which 50% of the enzyme is inhibited by the compound).

IC_{50} values of the compounds were calculated through GraphPad Prism Version 8 (GraphPad Software, La Jolla, California, USA) by a non-linear regression analysis utilising the relative fluorescence units (RFUs) measured throughout the assay. The initial velocities were calculated from the plotted RFUs, normalised (where 100% inhibition = control wells that contained no substrate and 0% inhibition = wells without inhibitor), and finally expressed as enzymatic activity.

6. Statistical Analysis

To perform statistical analysis, GraphPad Prism Version 8 was used. Data is represented as mean ± standard deviation. For comparison between the groups tested in the yield reduction assay, a one-way ANOVA with Dunnett´s test for multiple comparisons was used with 95% confidence intervals, where $P < 0.05$ was considered to be significantly different, and $P > 0.05$ was considered to not be significantly different.

Results

1. Compounds MP12, MP17, MP18, MP19 and MP21 show an IC_{50} of below 0.075 µM

To determine which of the novel compounds were active in inhibiting the SARS-CoV-2 protease, compounds were initially incubated with the recombinant enzyme at two different concentrations, 1µM and 0.1 µM. Compounds were incubated with the protease to allow for interaction between them, before FRET substrate was added and initial velocities were recorded by assessment with a plate reader. After this initial assessment of the compounds, six compounds were selected based on their inhibitory activity, and these were tested with the same assay at twelve different concentrations, ranging from 2 µM to 2 x 10^{-4} µM, which produced a dose-response curve from which the IC_{50} could be calculated.

Initial tests of the compounds with the enzymatic assay identified active compounds, *Figure 3*. A dose dependent inhibition can be observed, where higher concentrations of compound lead to higher percentage of inhibition of SARS-CoV-2 protease activity. Percentage inhibition of the compounds against SARS-CoV-2 protease can be seen in *Table 2*. Compounds MP7 and MP22 achieved the lowest levels of inhibition in the enzymatic assay, with 5.19% ± 12.49 and 1.75% ± 13.76 at 1 µM respectively, and 0% inhibition at 0.1 µM each. Compounds 11-acid, MP19 and CMP19 have reached approximately 100% inhibition at 1 µM of compound, with 100.69% ± 4.13, 101.63 % ± 4.37 and 104.52% ± 3.59 respectively.

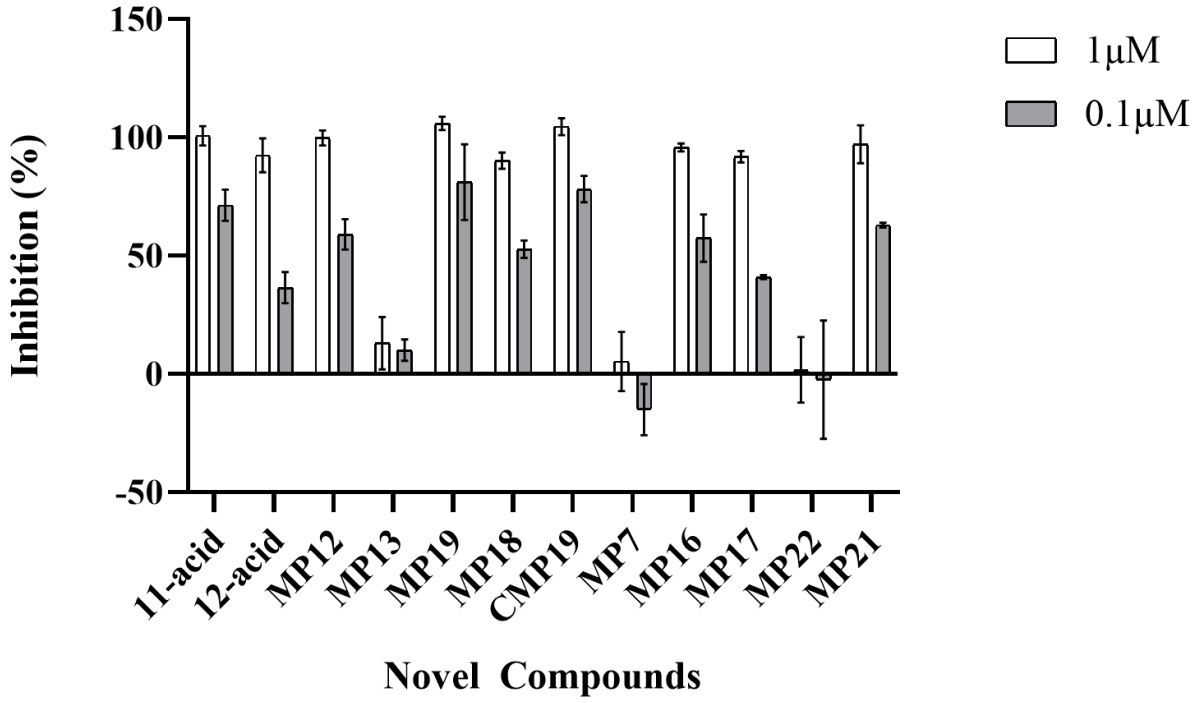


Figure 3. Percentage inhibition of the novel compounds against the SARS-CoV-2 3CLpro as assessed through the enzymatic FRET based assay. Novel compounds were tested at two concentrations, 1 µM (white) and 0.1 µM (grey), to determine which of the compounds were active in inhibiting the recombinant enzyme, and which were inactive. Active compounds include the compounds 11-acid, 12.acid, MP12, MP19, MP18, CMP19, MP16, MP17 and MP21. Inactive compounds are MP13, MP7 and MP22, due to their low percentage inhibition of the protease.

Table 2 Percentage inhibition of SARS-CoV-2 protease by novel compounds.

Compound	Percentage inhibition at 1µM (%)	Percentage inhibition at 0.1µM (%)
11 acid	100.69 ± 4.13	71.29 ± 6.62
12 acid	92.41 ± 7.21	36.48 ± 6.60
MP12	99.73 ± 3.12	58.94 ± 6.39
MP13	13.05 ± 11.06	10.09 ± 4.44
MP18	90.20 ± 3.49	52.76 ± 3.69
MP19	101.63 ± 4.37	57.06 ± 9.35
MP7	5.19 ± 12.49	-15.07 ± 10.77
CMP19	104.52 ± 3.59	78.15 ± 5.54
MP16	95.76 ± 1.69	57.44 ± 9.94
	91.83 ± 2.41	40.86 ± 0.83
MP17	1.75 ± 13.76	-2.47 ± 25.03
MP22	97.03 ± 8.02	62.95 ± 0.98
MP21		

After the initial screening, selected compounds were further tested to determine their IC_{50} through testing of 12 concentrations in the enzymatic assay, *Figure 4*.

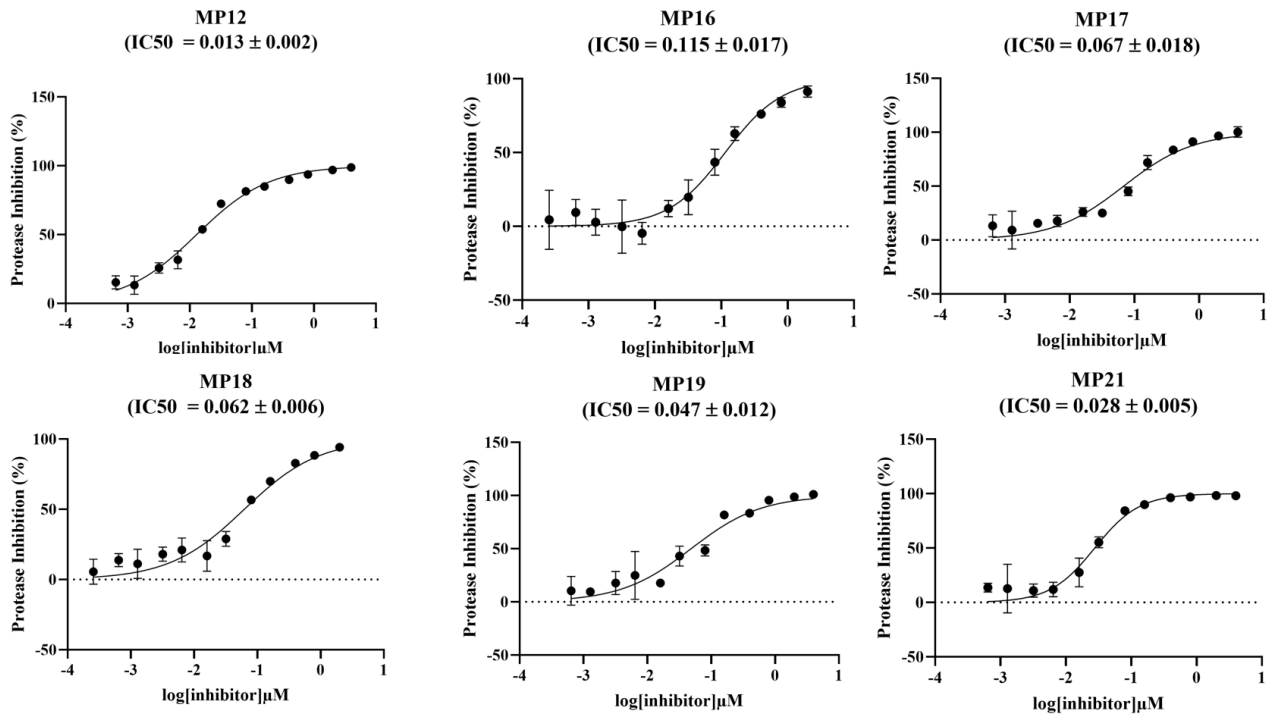


Figure 4. Dose response curves of selected compounds, which were assessed through FRET based enzymatic assays against the SARS-CoV-2 protease at concentrations ranging from 2 µM to 2 x 10^{-4} µM for creation of the dose response curves. Calculated curves allowed assessment of IC_{50} values for each compound, which were calculated with GraphPad Prism Version 8. Compounds with the lowest and most desirable IC_{50} values are MP12 (IC_{50} = 0.013 ± 0.002) and MP21 (IC_{50} = 0.028 ± 0.005), due to this indicating these compounds as being the most potent in inhibiting the viral protease. A table listing the IC_{50} values for each selected compound can be found in Appendix A.

The compound with the lowest IC_{50} was MP12, with a value of 0.014 µM, followed by MP21 (IC_{50} = 0.024 µM) and MP19 (IC_{50} = 0.044 µM). Tested compounds with the highest IC_{50} are MP16 (IC_{50} = 0.123 µM) and MP18 (IC_{50} = 0,074 µM).

2. Zika virus yield is highest 72 hours post infection

To determine at which point pI would yield the highest amount of Zika virus, cell culture flasks were infected with an MOI of 0.1, and supernatant was collected either at 48, 72 or 96 hours pI. Supernatant was then assessed through plaque assays to quantify virus titre. The three different time points were chosen, as those are the ones commonly recommended for Zika virus stock production.

Plaque assays of the supernatant taken at differing time points post infection show that taking supernatant at 72 hours post infection yields the highest concentration of virus. Zika virus was shown to be present at 1.175 x 10^6 PFU/ml after 72 hours, which was followed by stock collected after 96 hours of incubation, with a virus concentration of 1.0 x 10^6 PFU/ml, *Table 3.*

Table 3 Virus Stock Titration

Hours post infection	Virus concentration (PFU/ml)
48	2.2×10^{5}´
72	1.175×10^{6}
96	1.0×10^{6}

*for raw data, see Appendix B.

3. AEBSF presents cytotoxicity at the highest tested concentration, while no cytotoxicity is caused by Compound 104

Before yield reduction assays could be conducted, potential cytotoxicity of the compounds had to be assessed. This was done for both Compound 104 and compound AEBSF, which is a broad spectrum serine protease inhibitor that was used as a reference compound in these experiments. Cell viability was assessed through MTT assays, where HUH-7 cells were seeded and treated with differing concentrations of compound, 200µM and 20 µM for AEBSF and two-fold dilutions of Compound 104 ranging from 50 µM and 1.5625 µM for Compound 104. After addition of MTT solution and 10 % SDS, 0.01 M HCl solution at appropriate times and the required incubation periods, optical densities of the experiments were assessed with a plate reader and cytotoxicity was calculated.

The cell viability assay has shown that AEBSF causes cell toxicity at a concentration of 200 µM, *Figure 5-A.* Cell viability at this concentration was measured as 74.527% $\pm$ 1.876. Cell toxicity was not found at the lower concentrations of AEBSF. The MTT assay conducted for Compound 104 showed that there was no cell toxicity caused by any of the compound concentrations, *Figure 5-B.*

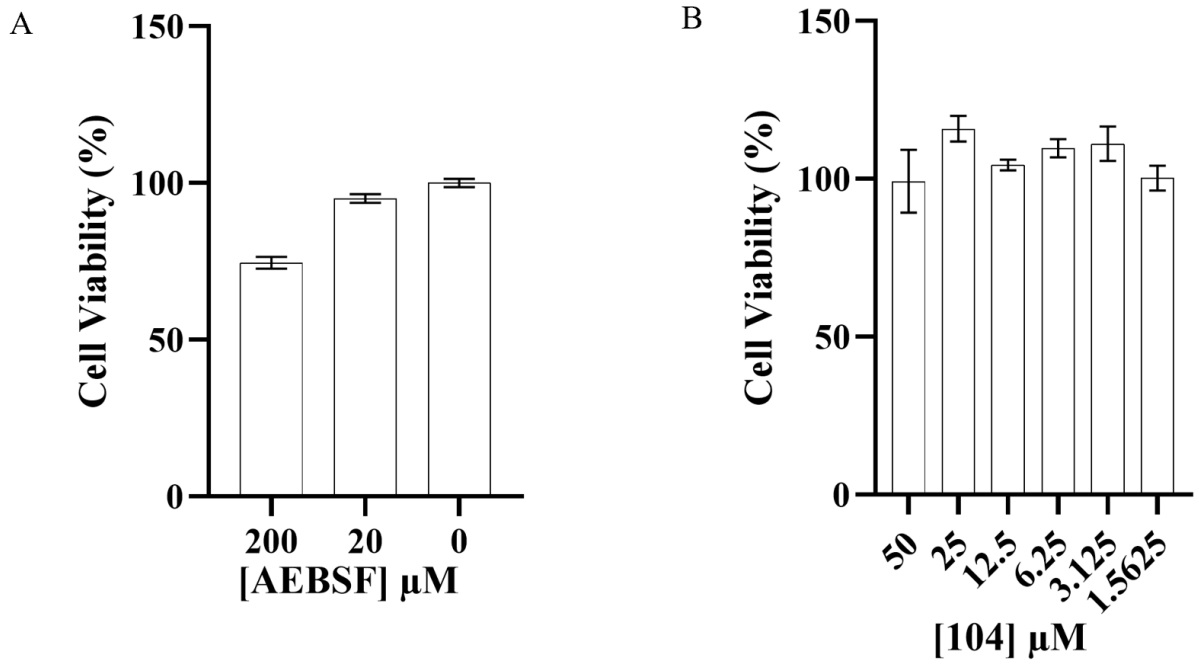


Figure 5. 5-A: Determined cytotoxicity of compound AEBSF through assessment by MTT assays. Cell viability percentage at 200 µM is 74.527% ± 1.876, at 20 µM is 95.048% ± 1.367 and at 0 µM is 100% ± 1.201, indicating that at the higher concentration, cytotoxicity is occurring due to the presence of the compound at 200 µM. At the lower concentration, cytotoxicity is not observed. 5-B: Determined cytotoxicity of Compound 104 after assessment by MTT assays. Cell viability percentage at concentrations of 50 µM is 99.238% ± 9.983, at 25 µM is 115.825% ± 4.063, at 12.5 µM is 104% ± 1.697, at 6.25 µM is 109.682% ± 2.859, at 3.125 µM is 111.085% ± 5.438 and at 1.5625 µM is 100.288% ± 3.938. This indicates that none of the tested concentrations of Compound 104 cause cell death in this assay.

4. Compound 104 gives inconclusive results under the tested conditions

To assess the inhibitory potency of virus replication by compounds AEBSF and Compound 104, yield reduction assays were used, followed by assessment of resulting virus titre after treatment with the different concentrations of the compounds through plaque assays. Yield reduction assays were performed by seeding HUH-7 cells and treating them with the beforementioned concentrations of the compounds, before infecting them with Zika virus. After incubation, supernatant was collected and virus titre was quantified through plaque assays.

Compound AEBSF shows total inhibition of Zika virus production at a concentration of 200 µM, despite causing some cytotoxicity at this concentration, Figure 6-A. At a concentration of 20 µM, PFU/ml values were halved when infected cells were treated with this concentration. In the case of Compound 104, the virus yield determined after the treatment with the compound showed that except for the 6.25 µM concentration, there was an overall reduction in virus production in the presence of Compound 104, *Figure 6-B*. Greatest inhibition was achieved at a concentration of 1.5625, however, there was no significant difference between 1.5625 µM, 12.5 µM and 25 µM. However, inhibition does not seem to be dose-dependent as can be observed in the case of AEBSF.

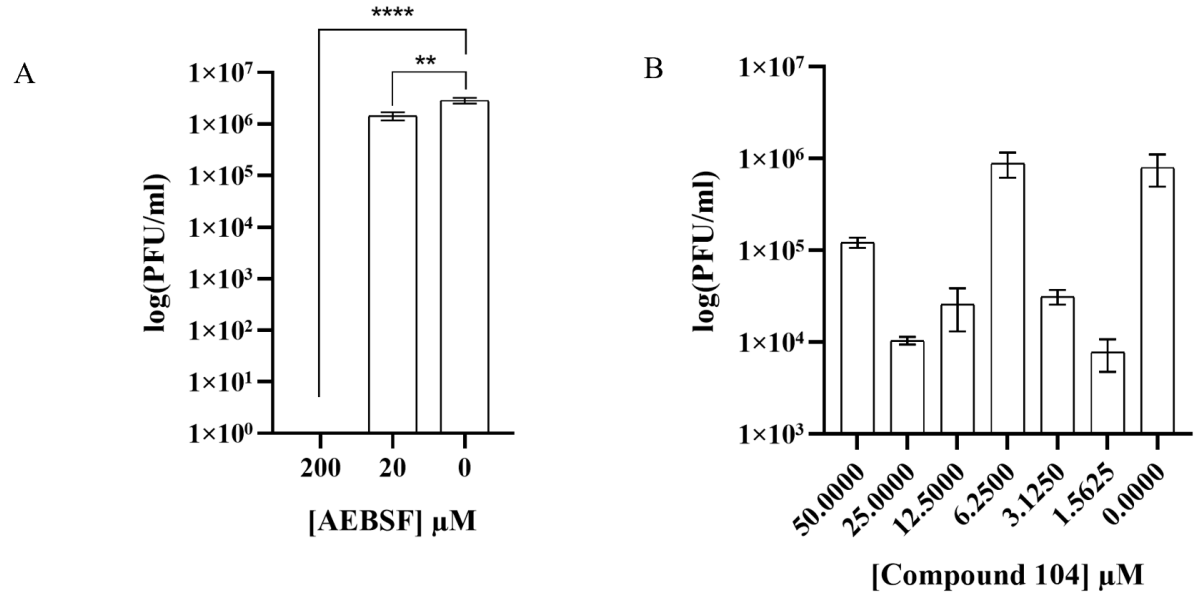


Figure 6. 6-A: Results of the yield reduction assay to determine the inhibition of virus production by the presence of AEBSF. AEBSF shows a dose-dependent inhibition of virus production. At 200 µM, inhibition of virus production compared to 0 µM is significant at $P < 0.0001$, and at 20 µM, inhibition is significant at $P < 0.0017$. Standard deviation is presented as error bars. 6-B: Results of the yield reduction assay to determine the inhibition

of virus protection by the presence of Compound 104. Unlike AEBSF, Compound 104 does not show dose-dependent inhibition of virus production. Least amount of inhibition was observed at a concentration of 6.25 µM, which gave comparable results to the control, where cells were not treated with any concentration of the compound. Standard deviation is presented through error bars. For values, see Appendix C

Discussion

1. Potential pan-coronavirus protease inhibitors

The current SARS-CoV-2 pandemic has shown the lack of preparedness for emerging viral diseases. While vaccines against SARS-CoV-2 are now available, the need for medications to treat primary SARS-CoV-2 infection still remains, especially as some part of the population will remain un-vaccinated, remaining vulnerable to a severe disease course (73).

In this study, novel compounds were tested in FRET based enzymatic assays for their inhibitory activity against the SARS-CoV-2 main protease. Of the tested compounds, the initial screening identified inactive compounds, including MP7 and MP22, as well as a compound with low activity, MP13. The remaining compounds showed activity in the assay. Compounds MP12, MP16, MP17, MP18, MP19 and MP21 were chosen for further testing based on their activity in the initial screening. The compounds were tested at different concentrations to create a dose-response curve that would allow the determination of their respective IC_{50} values. The lowest IC_{50} value was identified in MP12 (IC_{50} = 0.014 µM), followed by MP21 (IC_{50} = 0.024 µM), MP19 (IC_{50} = 0.044 µM), MP17 (IC_{50} = 0.054 µM), MP18 (IC_{50} =0.074 µM) and lastly MP16 (IC_{50} =0.123 µM). Generally speaking, the lower the IC_{50} of a compound, the more potent the drug (74). Additionally, it is thought that a lower IC_{50} decreases the chances of adverse side effects upon treatment (75).

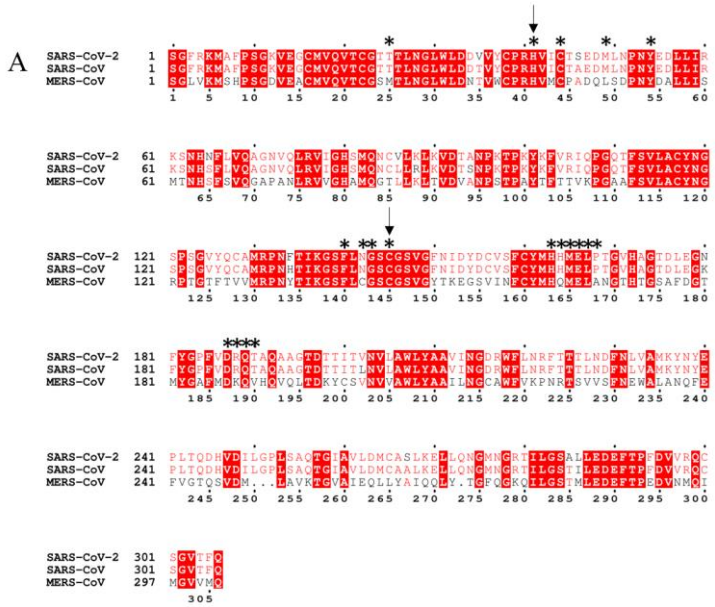
The data collected here presents some attractive candidates, with low µM IC_{50} values even comparable to those of pharmaceutical companies (76,77), and thus they should be seen as promising starting points for further medicinal chemistry. Further drug testing in cell culture based assays for their ability to cross the cell membrane and inhibit virus replication is needed. More so, the compounds will need to undergo more stages of drug development, to assess their potential of inhibiting the main protease *in vivo*, and also to assess potential harmful or toxic side effects the compounds may have.

Proof of concept for this type of drug lies in nirmatrelvir, a main protease inhibitor of SARS-CoV-2 developed by Pfizer, which, when taken within five days of primary SARS-CoV-2 infection, has been shown to reduce the risk of death by 89% of non-hospitalised high-risk adults with COVID-19 (78). Additionally to testing the novel compounds in cell based assays with SARS-CoV-2, it is of interest to test these also against MERS-CoV and one of the less pathogenic human coronaviruses, such as the HCoV-229E. If possible, these should be tested in enzymatic assays first, and then assessment in cell culture should take place, to assess their inhibitory potential against the other human coronavirus proteases first. MERS-CoV should be chosen due to its prominence compared to SARS-CoV, as cases of MERS-CoV still occur yearly. Additionally, the MERS-CoV active site is less conserved than the SARS-CoV active site when compared to SARS-CoV-2, with only 51% sequence similarity between their main proteases (45). The HCoV-229E would be an interesting target, as this would assess the

potential of the novel compounds not only against one of the less pathogenic human coronaviruses, but also against a coronavirus of a different genus, as HCoV-229E is an *alpha* coronavirus, whereas SARS-CoV-2, MERS-CoV and SARS-CoV are *beta* coronaviruses. Testing the novel compounds here against MERS-CoV and HCoV-229E would thus assess their pan-coronavirus inhibitory potential.

Protease inhibitors, as they are developed here, are a staple in the treatment of chronic viral infections, such as HIV and HCV (79,80). Using protease inhibitors in acute infections, as is the case in the treatment of SARS-CoV-2, requires the drugs to be taken either prophylactically, or directly after detection of the infection. As an exemplar, the use of nirmatrelvir is suggested within five days of symptom onset (55). Short term treatment with protease inhibitors may be possible without side effects that may develop during treatment of chronic infections (81).

The concept of a pan-coronavirus protease inhibitor has been mentioned multiple times by now. It is thought possible due to the high conservation of the 3CLpro between the coronaviruses (45–47,82,83), *Figure 7*, which has also been confirmed through crystal structure impositions (23,84). While amino acids may differ, or be replaced with amino acids from the same family, the catalytic dyad is conserved in all human coronaviruses.



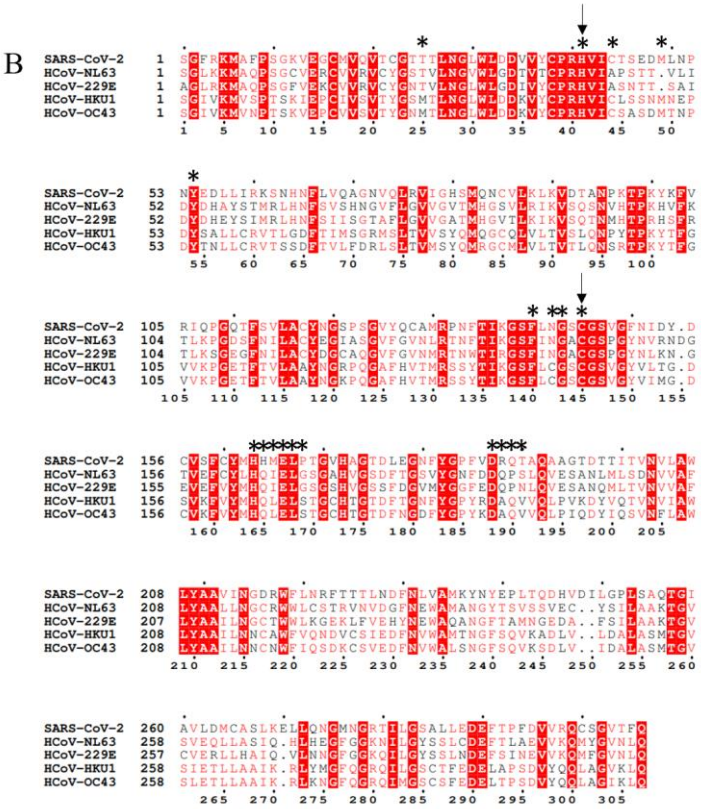


Figure 7. 7-A: Comparison of the 3CLpro sequences between the highly pathogenic human coronaviruses. Protein sequence alignment of the 3CLpro of SARS-CoV, SARS-CoV-2 and MERS-CoV, with shared residues between all three shaded in red, shared residues between two written in red, and distinct residues in white. Binding site residues of SARS-CoV-2 are indicated with an asterisk and the catalytic dyad is indicated with arrows. SARS-CoV-2 and SARS-CoV share a higher similarity to each other than SARS-CoV-2 and MERS-CoV do. 7-B: Comparison of the 3CLpro sequences between SARS-CoV-2 and the less pathogenic human coronaviruses. Protein sequence alignment of the 3CLpro of SARS-CoV-2, HCoV-NL63, HCoV-HKU1, HCoV-229E and HCoV-OC43, with shared residues shaded in red, shared residues between some or from the same amino acid family written in red, and distinct residues in white. Binding site residues of SARS-CoV-2 are indicated with an asterisk and the catalytic dyad is indicated with arrows. Sequence alignments were created with ESPript 3.0 (85).

Prior studies investigating potential pan-coronavirus protease inhibitors have shown that MERS-CoV only forms a weak dimer in the presence of a ligand (86) and favours different amino acids in the P2 position of the enzyme. The differences within the active site are thought to be one potential reason as to why SARS-CoV 3CLpro inhibitors did not show potent inhibition in MERS-CoV without structural modification (87). Between SARS-CoV-2 and MERS-CoV, the active sites of their 3CLpros differ in seven positions (88).

Lastly, drug resistance is always a possibility. The characteristically high mutation rate of RNA viruses has been known for a long time (89). However, in coronaviruses, the 3´-5´ exoribonuclease activity (ExoN) on nsp14 has been suggested to mediate proofreading during replication of these viruses (29,89). This ability has shown to be able to confer resistance against drugs such as nucleoside analogues such as remdesivir through excision of the compounds (29,30).

Even though this proofreading exists, mutations still occur, as can be seen in the latest SARS-CoV-2 variant Omicron, which has more than 60 mutations compared to the wild type strain

(90), which have been suggested to have evolved in an animal host after early divergence from other variants (90,91) before re-infecting humans (92), among other theories. Mutations within the Omicron variant have been identified in proteins as highly conserved as the 3CLpro. Prior research has identified 78 mutations within this enzyme, occurring both in the binding and the non-binding domain (54), although binding domain mutations were less common. Active site residues affected by the most common mutations occurring within the binding domain are Thr190, Met49, Thr25 and Arg188. Even though mutations have been identified in the SARS-CoV-2 3CLpro, the prevalence of the mutations in this gene is less than 5% compared to the number of mutations found in other proteins of the virus, such as the spike gene (44). Additionally, some mutations circulate with higher prevalence in Europe compared to other geographical regions. The 3CL-K90R mutation, for example, has a prevalence of 1.8% in Europe, while having been detected only in small amounts or not at all on other continents (44).

As SARS-CoV-2 continues to mutate, resistance against available treatments may develop, decreasing their effectiveness (73). If any of the already recorded mutations are able to confer resistance against active site protease inhibitors remains to be determined. Some mutations have been assessed against nirmatrelvir, *Table 4*, and thus far, no resistance has been shown.

Table 4 3CLpro mutations resistance tested against nirmatrelvir

Mutation	Variant	Resistance against nirmatrelvir *in vitro*	Source
3CL-K90R	B.1.351, B.1.351 + E516Q, B.1.351 + L18F, B.1.351 + P384L, B.1.351.2, B.1.351.3	Not compromised	(93)
3CL-P132H	BA.1, BA.2	Not compromised	(93)
		No reduced nirmatrelvir activity in biochemical assays	(94)
3CL-G15S	C.37, C.1.2, C.16. C.36 + L452R, C.36.3	Not compromised	(93)
3CL-T21I	B.1.1.318	Not compromised	(93)
3CL-L205V	P.2	Not compromised	(93)
3CL-L89F	B.1.2	Not compromised	(93)

Adapted from (93,95)

2. Yield Reduction assays for testing peptide hybrids in Zika Virus

Some flaviviruses have shown rapid spread after their introduction into the Western hemisphere (62), which has been attributed to ecological factors, such as the distribution and spread of vector mosquitoes, global warming and changes in urbanisation (66). Studies into the spread of Zika virus, specifically, have identified many countries to which Zika virus could potentially spread to (96), in addition to many other flaviviruses, which are already widely spread. Even though there are approximately 400 million infections caused by flavivirus each year (62), a pan-flavivirus drug has so far not been successfully developed, and there are doubts about this being possible, partially due to the cross-reactivity of the flavivirus immune sera (66).

In this study, the peptide hybrid Compound 104 was used to evaluate the method of yield reduction assays to test peptide hybrid compounds for drug development purposes in Zika virus. Prior unpublished internal experiments have shown that the use of MTT based cell viability assays are not suitable for testing peptide hybrid compounds against Zika virus. Compound 104 is a peptide hybrid that presents two positively charged residues that are thought to cause problems with cell permeability (97), which is the reason as to why the yield reduction assay was assessed as a potential method to assess these types of peptide hybrid compounds against Zika virus. Other, smaller peptide hybrids, such as those without charge, would present a higher degree of cell permeability, and thus not the whole category of peptide hybrids is difficult to assess. Many even possess a positive charge, due to the negative charge of the active site (98).

Here, MTT assays have been used to determine cytotoxic effect of the compounds and found no cytotoxic effect on HUH-7 cells caused by any of the concentrations at which Compound 104 was tested, *Figure 5*. Following this, a yield reduction assay was used to investigate virus production in presence of the compound. The results of this have shown a decrease in virus production, as assessed through plaque assays, *Figure 6*, except of at a concentration of 6.25 µM. As this result is rather unexpected, it would be of interest to repeat the experiment to confirm these results. Generally, yield reduction seems to have been achieved, however, one would expect a greater difference in virus yield between the different concentrations of compound. Thus, there should be further investigation into this assay as a way to test hybrid peptide compounds in Zika virus.

Prior studies of Compound 104 in other flavivirus proteases, through enzymatic assays, have shown the compound to have an IC_{50} of 0.176 µM in Dengue virus (97), 0.557 µM in West Nile Virus (97), as well as 0.25 µM in Zika virus, and 0.92 µM in Tick Borne Encephalitis Virus (99). Additionally, Compound 104 has been shown to be somewhat membrane permeable and achieved an EC_{50}, the concentration of a drug that induces half of the response, of 3.42 µM in Dengue Virus and 15.5 µM in West Nile Virus, which is very high in comparison to the IC_{50} values determined earlier. *In vitro* assessment with those two viruses has also shown a significant reduction of virus titre. In case of the yield reduction assay being an appropriate method to study peptide hybrids in Zika virus, this would allow the determination of the EC_{50} value of Compound 104, and thus comparison to those studies.

As a general discussion point, viral proteases are common antiviral targets. The flavivirus protease has been targeted during drug development before (100), but has so far proven to be a difficult drug target. Thus far, only very few non-peptidic compounds have been proven to

be active against this protease in Zika virus (101,102). Difficulties with targeting this enzyme are thought to lie in its relatively flat active site (62,68), which impedes drug binding. However, due to the well preserved active site of the flavivirus protease (68,70), drug development is ongoing.

Prior studies targeting the active site of the flavivirus NS2B-NS3 protease have been shown to be effective in *in vitro* studies, however, only poor success has been reported in *in vivo* assessments (68,99,103), where only one compound has been described to have activity against flaviviruses *in vivo* (102).

However, these difficulties also raise the question if the protease should be further targeted with active site inhibitors, or of allosteric inhibitors or even completely other viral drug targets might be a better choice for a pan-flavivirus drug. Prior studies have shown some *in vivo* efficacy on non-competitive inhibitors of the NS2B-NS3 protease (68). Allosteric inhibitors with good animal model results have been identified, and further chemical classes are now studied as potential allosteric inhibitors for the flavivirus proteases (104). Other viral targets that might be attractive drug targets for a potential pan-flavivirus compound include the RdRP and interestingly enough a transmembrane protein encoded by the NS4B (105). However, now, there have been new macrolides and uncharged peptide hybrids described (106,107), which suggest that the protease still is and will stay an attractive drug target.

Another important point that needs to be considered when targeting the NS2B-NS3 protease is the fact that his protease is a serine protease, which is similar to some host serine proteases (68), meaning that there is a chance of adverse effects when taking NS2B-NS3 protease inhibitors as treatment for flavivirus infection.

Conclusion

In general, the need for antivirals has become pronounced again by the current pandemic, and having a selection of antivirals will be useful in the future as novel viral diseases emerge.

However promising results, further research is needed for these compounds to be of any use in treating disease. For the coronavirus protease inhibitors, studying the ability to enter cells and inhibit virus production there is the next step that needs to be taken. Then, adverse reactions and possible drug-drug interactions need to be studied. If at least one of these compounds shows promising results and can be used as a protease inhibitor for the treatment of SARS-CoV-2 infection and potentially even other coronavirus caused infections, then this would be great for the treatment of many people suffering from disease and would decrease disease burden, as well as decreasing the chance of being without antivirals in case drug resistance develops against the ones we already have.

For Zika virus, the use of yield reduction assays as a way to study peptide hybrids should be further investigated, and if possible, the EC_{50} of Compound 104 should be assessed for comparison with known EC_{50} values of this compound in other flaviviruses.

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Appendix

Appendix A

Table A-1 IC_{50} values of tested compounds.

Compound	IC_{50} (μM)
MP12	0.014
MP16	0.123
MP17	0.054
MP18	0.074
MP19	0.044
MP21	0.024

Appendix B

Table B-1. Virus stock assessment through plaque assay

Hours post infection	10^{-3}		10^{-4}		10^{-5}		10^{-6}	
	Day 4	Day 5	Day 4	Day 5	Day 4	Day 5	Day 4	Day 5
48	64	N/A	23	38	0	1	0	0
	87	N/A	21	28	5	7	0	0
72	N/A	N/A	72	N/A	29	37	4	3
	N/A	N/A	88	N/A	22	21	2	0
96	N/A	N/A	41	59	1	6	2	3
	N/A	N/A	66	75	2	3	1	1

*N/A – too many plaques to count or plaques were fused

Appendix C

Table C-1 Yield Reduction caused by Compound 104

Compound	Concentration (μM)	PFU/ml
AEBSF	200	0
	20	1.40×10^6
	0	2.85×10^6
Compound 104	50	5.10×10^4
	25	1.04×10^4
	12.5	3.50×10^4
	6.25	8.86×10^5
	3.125	3.10×10^4
	1.5625	7.00×10^3
	0	8.0×10^5

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