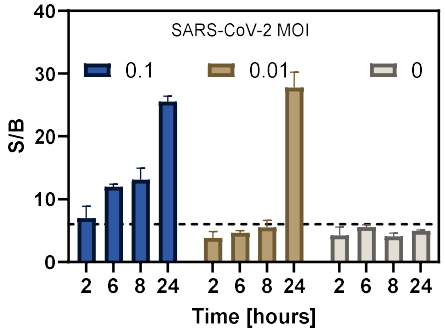
Application 1.

Time Course of SARS-CoV-2 PLPro Activity in lysates from Vero-E6 cells

Materials and Methods.

VeroE6 cells (ATCC, Manassas, VA, USA, Cat# CRL-1586) were grown to 90% confluency (approx. 3x 10^5 cells per well) in a 12-well plate with Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cells in 4 wells were infected with a MOI of 0.1 or 0.01 of the SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, Manassas, VA, USA) in viral growth medium (MEM + 2.5% heat inactivated fetal calf serum) and incubated at 37°C in a humidified incubator for 2, 4, 8, and 24 hours. Control cells were not infected and treated with medium. After each incubation time, MP Lysis Buffer (100µl) was added to washed monolayers and 25µl of lysates (corresponding to 75,000 cells) from each time point were combined with the PLpro Sensor in wells of a 96-well plate. The increases in relative fluorescence units (RFU) were measured in x2 replicates immediately and after 60 minutes in a fluorescence plate reader using excitation and emission wavelengths of λex=485nm and λem=528nm. The ratio of RFU between the 60-minute read and the initial read was calculated and plotted as Signal to Background (S/B) using GraphPad Prism software.

Results. Lysates infected with an MOI of 0.1 produced a S/B of 12 after 6 hours of incubation, compared to a S/B of 6 that was obtained in lysates from uninfected cells.[1] Lysates from cells that were infected with an MOI of 0.01 required an incubation time of approximately 24 hours to achieve a noticeable difference relative to uninfected cells. A S/B of 6 was used as a cutoff and is indicated by a dotted line in the figure.



Time course of SARS-CoV-2 PLpro cleavage activity on the protease sensor in lysates from VeroE6 infected cells.

Conclusions. These results correspond to the reported detection of intracellular SARS-CoV-2 virions ~10 hours post infection which require PLpro activity for assembly.[2]

Reference*:*

1. Publication preprint https://medrxiv.org/cgi/content/short/2024.05.21.24307588v1 PLOS One in press.
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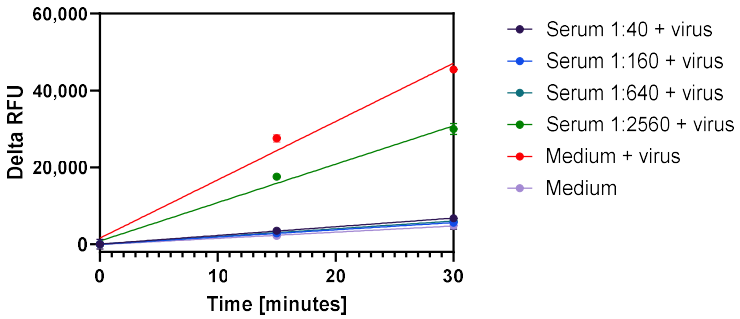
Application 2.

Detection of SARS-CoV-2 Neutralizing Antibody Activity in Lysates from Infected Vero E6 Cells.

Neutralization assays, such as plaque reduction neutralization test (PRNT), fluorescent antibody virus neutralization (FAVN) assays and immunological assays, play a pivotal role in evaluating the efficacy of vaccines, selecting convalescent plasma for clinical trials and therapeutic use, and defining immune evasion.[1] However, those assays require several steps and days to complete. In contrast, measuring SARS-CoV-2 PLpro activity as a marker of infection with Mesa Photonics Protease sensor will allow to more rapidly determine the efficacy of therapeutic or vaccine-derived antibodies than is possible with current tests. The possibility of the PLpro sensor to detect variants will play an important role for detecting new neutralizing antibodies that are urgently needed to combat the loss of efficacy of previously effective monoclonal therapeutics due to SARS-CoV-2 variants, as manifested by increased numbers of breakthrough infections in vaccinated individuals.[2-6]

A neutralization assay was conducted with Mesa Photonics protease sensor that demonstrates that PLpro cleavage activity is a useful biomarker for rapidly determining antibody neutralization activity. [7]

Materials and Methods. Patient serum was diluted 1:40, 1:160, 1:640 and 1:1250 in viral growth media (MEM + 2.5% heat inactivated fetal calf serum) and 200µl was added to an equal volume of 0.01 MOI SARS-CoV-2 USA-WA1/2020 in viral growth medium for 2 hours. Controls were medium with SARS-CoV-2 without serum and medium without virus. The mixtures (400µl) were added to 90% confluent VeroE6 cells in 12-well plates and the cells were incubated at 37°C in a humidified incubator for 24 hours. This mixture was then added to VeroE6 cells and lysates were prepared after 24 hours of incubation. The cell-based protease assay was performed using the PLpro Sensor that was added in 2x assay buffer for a final concentration of 50nM. The increase in relative fluorescence units (RFU) from peptide cleavage was monitored every 15 minutes for 30 minutes at room temperature in a fluorescence plate reader using excitation and emission wavelengths of λ_{ex}=485nm and λ_{em}=528nm. The difference in RFU between the 30-minute read and the initial read was calculated and plotted as Delta RFU using GraphPad Prism software.



Neutralization assay using PLpro peptide cleavage as a marker of infection in lysates from VeroE6 cells infected cells.

Results. Lysates from cells that were challenged with virus pretreated with serum dilution from 1:40, 1:160, and 1:640 produced fluorescence that was not distinguishable from the delta RFU obtained from uninfected control lysates (Medium), demonstrating complete neutralization. In contrast, lysates from cells in which SARS-CoV-2 had been incubated with the serum dilution of 1:2,560 produced delta RFU that was 33% lower than the delta RFU from untreated virus (Medium + virus), indicating that infection, albeit at a lower level, had occurred.

References

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7 Publication preprint https://medrxiv.org/cgi/content/short/2024.05.21.24307588v1 [PLOS One in press]

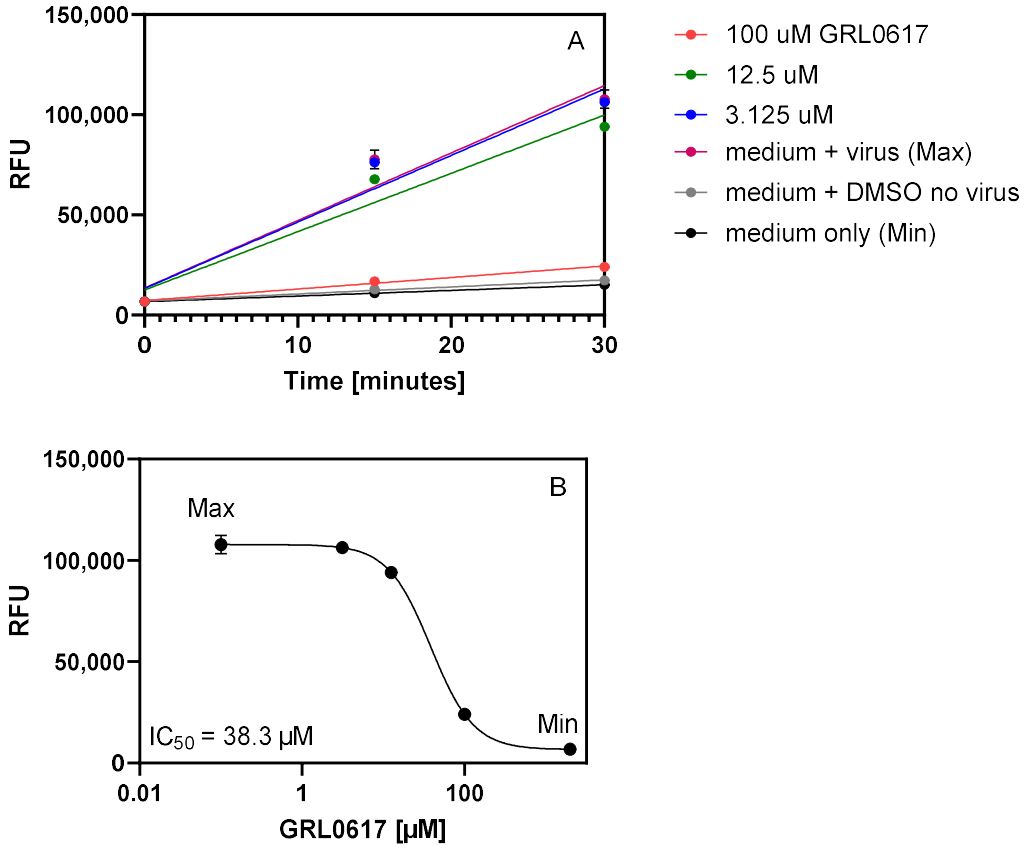
Application Note 3. Detection of SARS-CoV-2 PLpro inhibitors in Cellular Lysates

The detection and development of new SARS-CoV-2 antivirals is highly relevant because to date only 2 oral antiviral drugs are approved for treatment of COVID-19 in the United States: Nirmatrelvir (a component in Paxlovid) that targets MPro, and molnupiravir, a nucleoside analog developed by Merck targeting RNA-dependent RNA polymerase (RdRp).[1,2] Over the past 3 years variants with mutations in MPro have caused drug resistance under therapeutic pressure while concerns have been raised about molnupiravir's capacity to trigger SARS-CoV-2 mutations.[3–5] It is therefore important to study additional therapeutic targets, such as SARS-CoV-2 PLpro. Inhibiting the dual functions of PLpro—suppression of the innate immune response by cleaving ISG15 and processing of the viral polyprotein—have prompted numerous drug development efforts, but to date, none of the promising PLpro drug candidates have advanced to animal models.[5-7] One explanation is that most biochemical studies examined only the minimal proteolytic domain of PLpro without considering the other domains in SARS-CoV-2 proteins that influence PLpro activity and function. Although this limitation is overcome by tests that measure the extent of drug-induced cytopathic effect (CPE) in cells or quantify the amount of viral RNA in response to inhibition, these phenotypic assays take days to complete and require several user steps. Plaque reduction assays are used to measure the plaque-forming efficiency of a virus in the presence of inhibitor take 5-7 days. Assays using genetically modified SARS-CoV-2 strains or replicons are faster and simpler but are suitable only for the virus strains they have been established for.[8] In contrast, the SARS-CoV-2 PLpro assay described here is a simple add-and-measure homogeneous assay that can be scaled up to run in 96-well or 384 well plates. Time course data demonstrates that SARS-CoV-2 PLpro activity or inhibition can be detected in as little as 6 hours after infection with 0.1 MOI (Application Note 1) and testing can be completed in one working day with minimal user steps.[9]

Material and Methods. The well-characterized PLpro inhibitor GRL0617 (R&D Systems) was diluted in MEM without fetal calf serum to achieve concentrations between 2µM-100µM and 100µl was added to near confluent VeroE6 cells in wells of a 24-well plate. Cells were incubated for 2 hours at 37°C in a humidified incubator, washed with 1x PBS, and 0.01 MOI of SARS-CoV-2 USA-WA1/2020 was added in viral growth medium (MEM + 2.5% heat inactivated fetal calf serum). Controls included medium with SARS-CoV-2 with and without DMSO (1% for a final concentration of 0.25%) and medium only with and without DMSO. Cells were incubated at 37°C in a humidified incubator for 24 hours. Cell-based protease assays were performed using the PLpro protease sensor at a final concentration of 50nM final concentration. The increase in relative fluorescence units (RFU) from peptide cleavage in x2 replicates was monitored every 15 minutes for 30 minutes at room temperature in a fluorescence plate reader using excitation and emission wavelengths of λex=485nm and λem=528nm. The mean RFU after 30 minutes in lysates from cells pretreated with GRL0617 and Max (medium plus virus) and Min (medium without virus) values were plotted and the IC_{50} calculated using GraphPad Prism, nonlinear regression, variable slope. Some error bars are within the symbols.

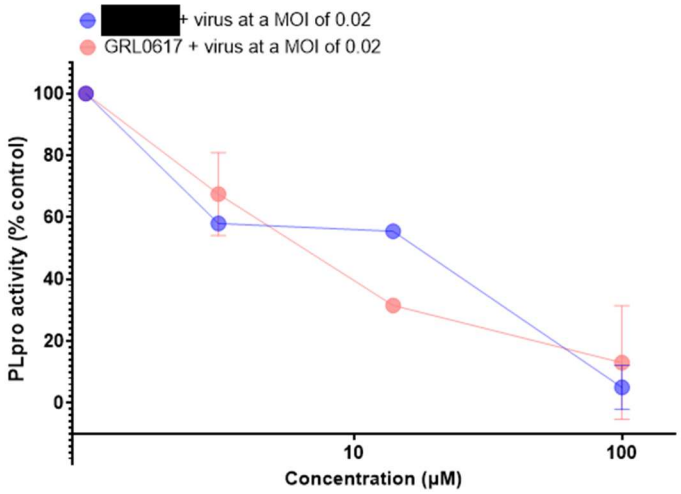
Results. The results shown in the Figure demonstrate that a concentration of 100µM of the inhibitor reduced the fluorescence to nearly the background fluorescence of the wells containing uninfected lysates (medium only or medium + 0.25% DMSO without virus), while

lower concentrations of inhibitor caused only a slight decrease in fluorescence relative to the controls (medium, medium + virus, and medium + DMSO no virus).
The tentative IC_{50} value of 38.3µM obtained for GRL0617 in our hands was within one order of magnitude of the EC_{50}=21 ± 2 µM reported in a virus proliferation assay, demonstrating successful inhibition.[11]



GRL0617 inhibition of SARS-CoV-2 PLpro peptide cleavage in lysates from VeroE6 infected cells.

Further, the PLpro sensor was successfully used in an independent study to demonstrate PLpro inhibition of a new, proprietary inhibitor [redacted].



Inhibition of SARS-CoV-2 PLpro peptide cleavage in lysates from infected Vero-CCL81 cells pre-treated with GRL0617 or a proprietary inhibitor (redacted).

Credits: Edmarcia Elisa de Souza and Carsten Wrenger University of São Paulo, Brazil and Christian Betzel, University of Hamburg, Germany.

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10 Publication preprint https://medrxiv.org/cgi/content/short/2024.05.21.24307588v1 (PLOS One in press).

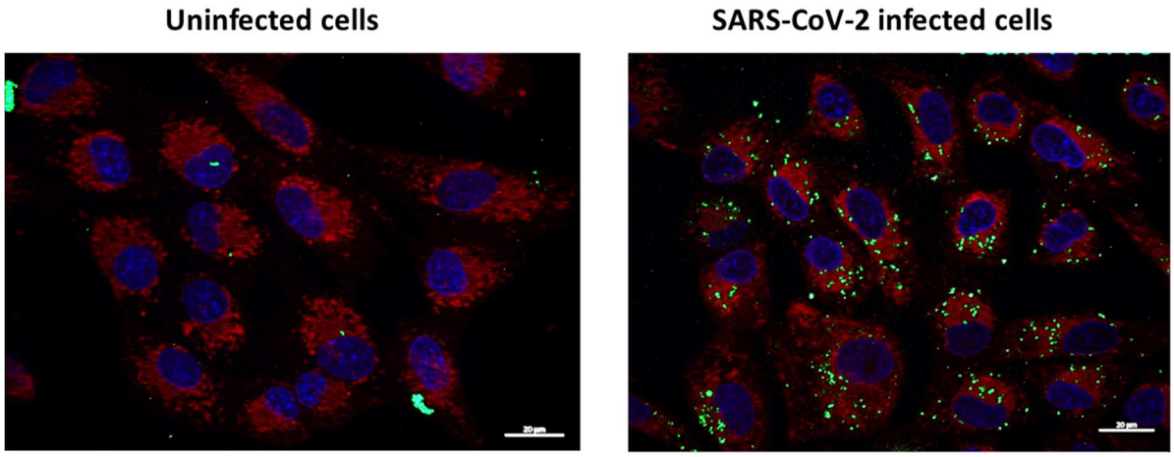
Application Note 4. Imaging SARS-CoV-2 PLpro Activity in Live Cells

The effects of detergents and reagents in the lysis buffer that might interfere with assay performance and inhibitor binding can be avoided by monitoring the cleavage of the PLpro sensor in whole, undisrupted cells infected with SARS-CoV-2. This was demonstrated for the first time by Edmarcia Elisa de Souza and Carsten Wrenger University of São Paulo, Brazil and Christian Betzel, University of Hamburg, Germany.

Material and Methods

Vero-CCL81 was cultured in a glass-bottom 4-wells tissue culture plate (Greiner Bio-One) in complete medium with 5% CO2 at 37°C for 24 hours to reach a confluence of approximately 80% and infected with Wuhan SARS-CoV-2 at MOI of 0.02. Cells were stained with Hoechst 33342 (Thermo Fisher Scientific - H1399) at 10µM, Cell Tracker Deep Red (Thermo Fisher Scientific - C34565) at 1 µM, and 300 nM PLpro Protease Sensor into PBS 1x for 10 min in 5% CO2 at 37°C. Subsequently, the cells were washed 3x with PBS 1x and fluorescence imaged using a ZEISS AxioObserver Z1 equipped with ApoTome2 and 5% CO_2 at 37°C incubation chamber and a ×63 oil-immersion objective. The entire fixed cell volume was projected as 2D image by orthogonal projection in the frontal X/Y directions from Z stacking images or displayed as 3D image using Zen 2.6 blue edition software (ZEISS).

The GMP editor was used to align the overlay with green at 50% opacity, green was saturated, with background black, then the black background was removed using an alpha layer to make it transparent. Lastly, the red-blue layer behind the green layer was turned on to show through the transparency (courtesy of Anthony Gomez, Mesa Photonics).



Live cell monitoring of SARS-CoV-2 Protease Sensor cleavage in infected VeroE6 cells.