

Detecting SARS-CoV-2 3CLpro expression and activity using a polyclonal antiserum and a luciferase-based biosensor

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ABSTRACT

The need to stem the current outbreak of SARS-CoV-2 responsible for COVID-19 is driving the search for inhibitors that will block coronavirus replication and pathogenesis. The coronavirus 3C-like protease (3CLpro) encoded in the replicase polyprotein is an attractive target for antiviral drug development because protease activity is required for generating a functional replication complex. Reagents that can be used to screen for protease inhibitors and for identifying the replicase products of SARS-CoV-2 are urgently needed. Here we describe a luminescence-based biosensor assay for evaluating small molecule inhibitors of SARS-CoV-2 3CLpro/main protease. We also document that a polyclonal rabbit antiserum developed against SARS-CoV 3CLpro cross reacts with the highly conserved 3CLpro of SARS-CoV-2. These reagents will facilitate the pre-clinical evaluation of SARS-CoV-2 protease inhibitors.

1. Introduction

The emergence of SARS-CoV-2 in 2019 has resulted in a pandemic with enormous impact on the human health and the global economy. SARS-CoV-2 is a member of the *Coronaviridae* family, which contains over 200 related viruses. Thus, there is a need to develop strategies to identify inhibitors that block highly conserved viral proteins essential for virus replication. The coronavirus 3C-like protease (3CLpro, also termed main protease, Mpro) is such a target (He et al., 2020; Li and Kang, 2020). This protease cleaves the replicase polyprotein at 11 sites, and this processing is required to generate a functional replicase complex. Multiple antiviral drug candidates targeting SARS-CoV-2 3CLpro have been described and are currently under evaluation for their ability to reduce viral replication and pathogenesis (Dai et al., 2020; Hattori et al., 2020; Jin et al., 2020; Rathnayake et al., 2020; Zhang et al., 2020). Evaluating inhibitors of SARS-CoV-2 requires Biosafety Level 3 (BSL-3) facilities, which are inaccessible to most of the scientific community, highlighting the need to develop assays that can be applied in a BSL-2 setting.

To assess SARS-CoV-2 3CLpro activity, we adapted a luminescence-based biosensor that we previously used for evaluating MERS-CoV 3CLpro activity (Kilianski et al., 2013). This biosensor is based on a circularly permuted version of firefly luciferase which is held inactive by a flexible linker (Galbán et al., 2013). The insertion of the 3CLpro target site (VRLQS) in the linker region allowed for cleavage by the protease, resulting in a conformational change in the protein that led to generation of bioluminescence. We reported that this 3CLpro biosensor activity was inhibited by a small molecule that bound to the active site of the MERS-CoV protease.

Here we describe the utility of this luminescence-based biosensor to evaluate the inhibitors of SARS-CoV-2 3CLpro. This assay can be used in a BSL-2 laboratory. We also document that a rabbit antiserum developed against SARS-CoV 3CLpro cross reacts with highly conserved SARS-CoV-2 3CLpro and that this antiserum can be used in immunofluorescence and western blotting assays. We are making these reagents available to the research community with the hope that they will facilitate the discovery and characterization of small molecule inhibitors against SARS-CoV-2.

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2. Results

SARS-CoV-2 3CLpro activates the pGlo-VRLQS biosensor. To determine if the SARS-CoV-2 3CLpro activity could be assessed using an established biosensor assay, we generated a plasmid that expressed the nsp4, nsp5 and the amino terminal part of nsp6, termed pp3CLpro (Fig. 1A). Our previous studies showed that expressing this coronavirus polyprotein allows for autocatalytic processing and release of 3CLpro. The released enzyme can then cleave at the conserved sequence (VRLQ/S) in the biosensor causing its activation (Fig. 1B) (Kilianski et al., 2013). We also generated an inactive mutant (C3408A) of 3CLpro to determine if the protease's catalytic activity is required for biosensor activation. We found that transfecting increasing amounts of the pp3CLpro plasmid DNA into cells containing the biosensor resulted in a dose-dependent increase in the luciferase activity (Fig. 1C and Supplemental Tables 1 and 2). In contrast, no signal was detected when the catalytically inactive form of 3CLpro was used, suggesting that the enzymatic activity of the protease was essential for biosensor activation. Of note, the activity of the SARS-CoV-2 3CLpro was similar to the one we previously reported for 3CLpro of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Kilianski et al., 2013).

3CLpro inhibitor blocks biosensor activation as well as SARS-CoV-2 replication. To determine if our biosensor assay could be used to evaluate potential inhibitors of 3CLpro, we tested a commercially available compound (GRL-0496) previously documented to inhibit 3CLpro activity of SARS-CoV (Ghosh et al., 2008). We found that GRL-0496 caused a dose-dependent inhibition of 3CLpro from

SARS-CoV, SARS-CoV-2 and MERS-CoV, when tested with our biosensor assay (Fig. 2A). The compound also inhibited SARS-CoV-2 replication in Vero cells in a dose-dependent manner (Fig. 2B). Remarkably, the half-maximal inhibitory concentration (IC_{50}) of GRL-0496 in the biosensor assay (3.41 μ M) was highly similar to that obtained in the virus replication assay (3.29 μ M). These results strongly suggest that the biosensor assay can be reliably used to screen for potential inhibitors of SARS-CoV-2 3CLpro.

Antiserum generated against SARS-CoV 3CLpro recognizes SARS-CoV-2 3CLpro. Bioinformatic analysis of SARS-CoV-2 3CLpro revealed that the protease has 96% identity with the 3CLpro of SARS-CoV (Fig. 3A). This led us to hypothesize that antibodies generated against SARS-CoV 3CLpro may cross react with SARS-CoV-2 3CLpro. To test this hypothesis, we evaluated a rabbit polyclonal antiserum that we had previously raised against 3CLpro of SARS-CoV for its ability to detect the SARS-CoV-2 protease by immunofluorescence (IF) and Western blot. The IF analysis of SARS-CoV-2-infected Vero cells with an anti-dsRNA antibody and the anti-3CLpro antiserum yielded a strong and overlapping signal, suggesting optimal performance of the antiserum (Fig. 3B). The typical punctate, perinuclear staining we observed in the infected cells was consistent with the previous reports describing the distribution of SARS-CoV replicase proteins (Prentice et al., 2004). To test the antiserum for Western blot, we used lysates from SARS-CoV-2-infected Caco-2 ACE2/TMPRSS2 cells harvested at 8, 12 or 24 h post-infection. The antiserum recognized a protein of 26 kDa, consistent with the expected size of nsp5/3CLpro (Fig. 3C). We also noted a higher molecular weight smear about 130 kDa, which likely

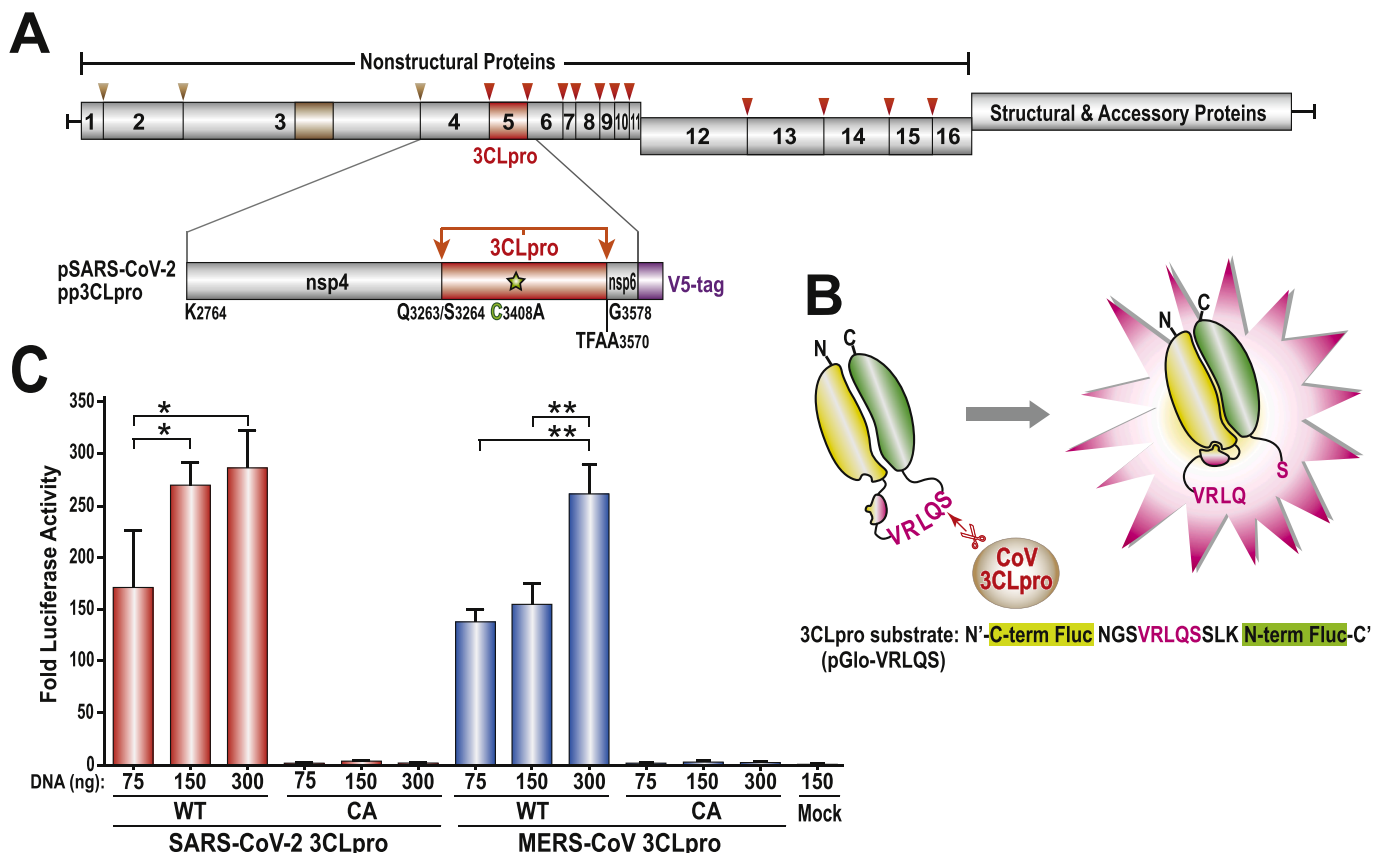


Fig. 1. Evaluating SARS-CoV-2 3CL protease (3CLpro) activity using a luciferase-based biosensor. A) Diagram of the region of SARS-CoV-2 nonstructural proteins 4, 5 and the amino-terminal region of nsp6 that was cloned into pcDNA3.1 expression vector with an in-frame V5 epitope tag. B) Schematic diagram of the pGlo-VRLQS biosensor that is activated upon cleavage by 3CLpro (Kilianski et al., 2013). C) Dose-dependent response of the biosensor after transfection of the indicated amount of plasmid DNA expressing the SARS-CoV-2 3CLpro or the MERS-CoV 3CLpro. Fold changes of luciferase activity over empty vector control are plotted (mock). Data are representative of three independent experiments performed in triplicate and presented as means $\pm$ SD. WT, wild type. CA, catalytic mutant (C3408A). Statistical analysis of the data was performed using the one-way ANOVA F-test, *, $p < 0.05$; **, $p < 0.001$.

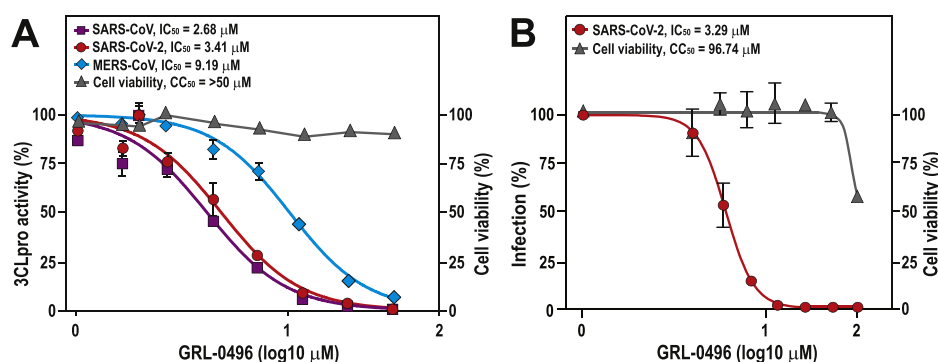


Fig. 2. Evaluating 3CL-protease inhibitor GRL-0496 using the luciferase biosensor and in SARS-CoV-2 infected cells. A) Dose-dependent inhibition of SARS-CoV, SARS-CoV-2 and MERS-CoV 3CLpro activity by GRL-0496 using the pGlo-VRLQS biosensor assay. HEK-293 cells were transfected with renilla luciferase control plasmid DNA, the 3CLpro-VRLQS substrate, and the indicated amount of plasmid DNA expressing 3CLpro. GRL-0496 was added at 14–16 h post-transfection, and cells harvest 6 h later, and evaluated for luciferase activity as indicated in the Methods. B) Dose-dependent inhibition of SARS-CoV-2 replication by GRL-0496 in Vero E6 cells. VeroE6 cells were infected with SARS-CoV-2 and GRL-0496 added at 1 h post-infection. Cells were harvested at 48 h post-infection and evaluated for expression of nucleocapsid protein using an immunofluorescence assay described in Methods. Data are representative of 2–3 independent experiments and presented as the mean $\pm$ SD. The half maximal inhibitory concentration (IC₅₀) values and the half maximal cytotoxic concentration (CC₅₀) were analyzed by GraphPad Prism 8 and verified SAS version 9.4 software.

includes forms of the precursor polyprotein that is processed to generate mature 3CLpro/nsp5. We further evaluated the specificity of the antiserum using HEK 293T cells transfected with the pp3CLpro plasmid. As expected, the antiserum detected both the polyprotein precursor containing 3CLpro (nsp4/5/6*) and the cleaved form of 3CLpro (nsp5/6*) (Fig. 3D). To confirm these results, we probed the Western blot membranes with an antibody against the V5 tag appended at the C-terminal end of the protein. This yielded a banding pattern consistent with that obtained with the anti-3CLpro serum, indicating that the antiserum can be used for detecting protease expression in both infected and transfected cells.

3. Discussion

We describe a luciferase-based biosensor assay for evaluating small molecule inhibitors of SARS-CoV-2 3CLpro. Several protease inhibitors have recently been reported to inhibit the replication of SARS-CoV-2 in cell culture (He et al., 2020; Li and Kang, 2020). However, for these compounds, it remains to be determined if they exert their antiviral activity by inhibiting a viral or a host protease. Our cell-based biosensor represents a powerful tool to evaluate the protease specificity of these compounds. Given that the biosensor assay yielded a similar IC₅₀ for GRL-0496 as the virus replication assay, the biosensor can serve as a great surrogate model to examine the antiviral efficacy of drug candidates. We have already begun to share the biosensor and other related reagents with laboratories around the world. Indeed, the biosensor assay was recently used to verify that an antiviral drug that inhibits replication of SARS-CoV-2 targets 3CLpro (Drayman et al., 2020).

Our luciferase-based biosensor assay complements a recently described FlipGFP-based biosensor assay (Froggatt et al., 2020). Similar to the luciferase reporter, the FlipGFP reporter fluoresces only after cleavage by 3CLpro. The FlipGFP reporter was shown to be cleaved by 3CLpro of multiple alpha- and beta-CoVs and can be used in high throughput assays for screening antiviral drugs. These two independent assays will be useful for validation of candidate compounds identified in high throughput screens.

Our finding that a polyclonal antiserum generated against SARS-CoV 3CLpro recognizes SARS-CoV-2 3CLpro is expected, as the two proteins are 96% identical. We are currently evaluating other antisera generated against SARS-CoV nonstructural proteins for cross reactivity with SARS-CoV-2. The biosensor assay and the polyclonal antiserum are freely

available to scientist around the world to facilitate research on SARS-CoV-2 and stop the on-going pandemic.

4. Materials and methods

SARS-CoV-2 3CLpro expression plasmid. The nucleotide sequence of the SARS-CoV-2 nonstructural protein 4, 5 and the N-terminal part of nsp6 (ORF1a amino acids 2764–3578; GenBank accession number MN908947.1) was codon optimized, synthesized (GenScript), and cloned into pcDNA3.1-V5/His-B (Invitrogen) to generate pp3CLpro. A catalytically inactive mutant (in which the catalytic cysteine 3408 was changed to alanine, designated CA) was generated using site-directed mutagenesis (Supplementary Table 3). The complete sequence of the plasmid pp3CLpro is provided in the supplementary information.

Biosensor expression plasmid and biosensor assay. The pGlo-30F-VRLQS biosensor is a circularly permuted Photuris pennsylvanica luciferase optimized for expression in cell culture and contains the 3CLpro cleavage site (Galbán et al., 2013; Kilianski et al., 2013). To evaluate 3CLpro activity, HEK 293T/17 cells (ATCC® CRL-11268™) were plated into CellBind 24-well plate (Corning, #3337) at a density of 1.5×10^5 cells per well and incubated at 37 °C for 20–24 h. The cells were transfected with 150 ng pGlo-30F-VRLQS, 10 ng pRL-TK (Promega), and increasing amounts of protease expression plasmid. Transfections were equalized to 300 ng per well with an empty pcDNA3.1-V5/His-B vector using TransIT-LT1 transfection reagent (Mirus). At 20 h post-transfection, the cells were lysed with 150 μ l passive lysis buffer (Promega) and 25 μ l of lysate was assayed for luciferase activity using 96-well white-bottom assay plates (Corning) and dual-luciferase activating reagents (Promega).

3CLpro inhibitor. GRL-0496 (Focus Biomolecules, #10-4960-0005) was previously shown to inhibit SARS-CoV 3CLpro (Ghosh et al., 2008). The compound was dissolved in dimethyl sulfoxide (DMSO) and used at the indicated concentrations.

Evaluation of GRL-0496 in SARS-CoV-2 infected cells and biosensor expressing cells. Vero E6 cells were seeded into poly-L-Lysine-coated 96-well plates at a density of 1.5×10^4 cells per well. The next day, the cells were infected with SARS-CoV-2 of a multiplicity of infection of 0.1 for 1 h, and GRL-0496 was added at 2-fold serial dilutions ranging between 1.56 and 100 μ M. DMSO served as a negative control. Forty-eight hours later, the cells were fixed in 4% paraformaldehyde and processed for immunofluorescence as previously

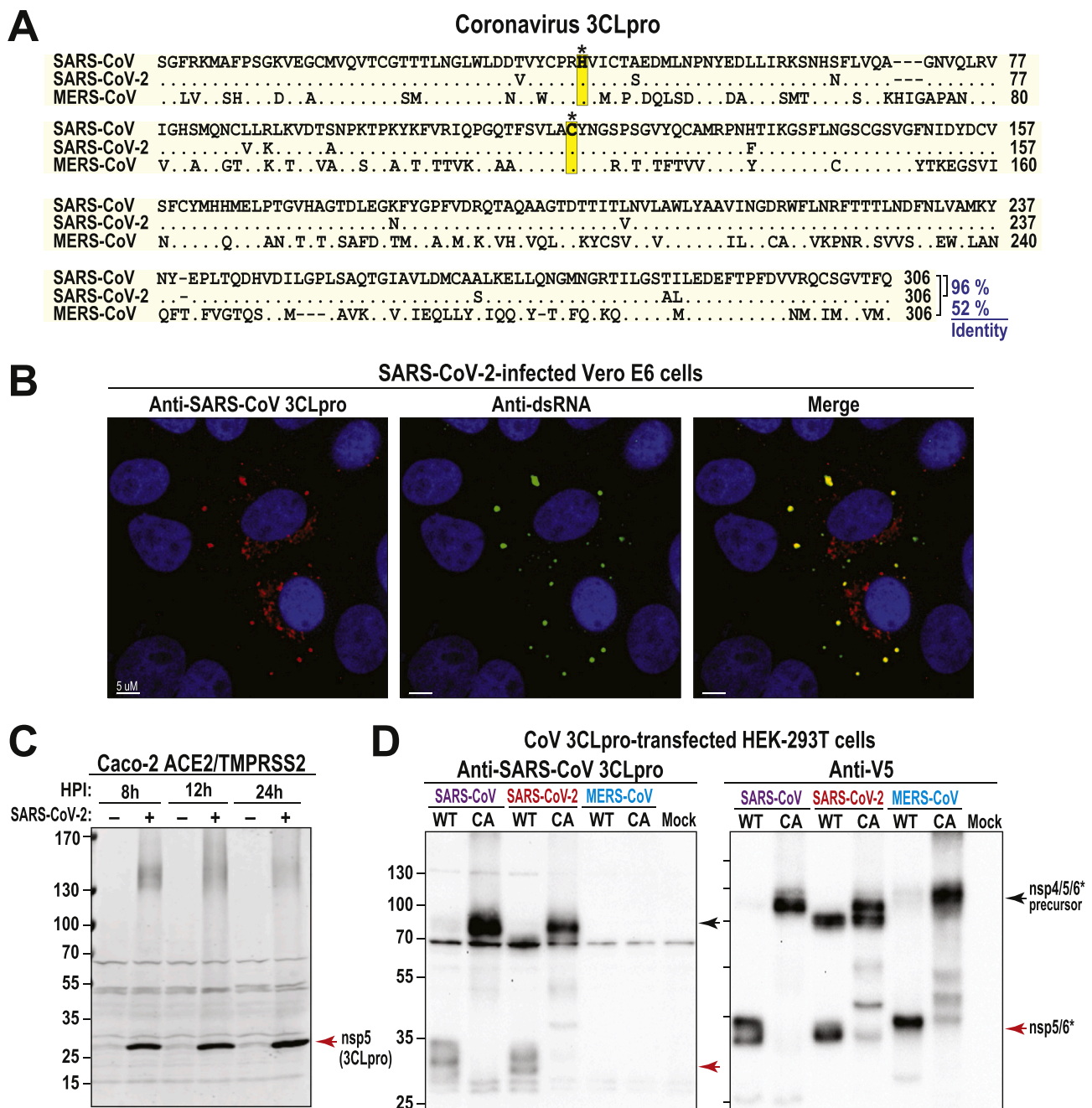


Fig. 3. Antiserum generated against SARS-CoV 3CLpro recognizes SARS-CoV-2 3CLpro in immunofluorescent and Western blot assays. A) Amino acid alignment of 3CLpro from SARS-CoV (Urbani, AY278741.1), SARS-CoV-2 (Wuhan, MN908947.1) and MERS-CoV (EMC/2012, JX869059.2). *, catalytic histidine or cysteine. B) Immunofluorescent images of SARS-CoV-2-infected Vero E6 cells stained at 12 h post-infection with anti-3CLpro antiserum (left), anti-dsRNA antibody J2 (middle), and DAPI (blue), with the merged image on the right. C) Western blot detection of nsp5 (3CLpro) of SARS-CoV-2-infected Caco-2 ACE2/TMPRSS2 cells at 8, 12 and 24 h post-infection (HPI) using anti-SARS-CoV 3CLpro antiserum. D) Western blot detection of 3CLpro precursor, nsp4/5/6*, and its cleavage products, nsp5/6*, in transfected HEK 293T cells using the anti-3CLpro antiserum (left) and anti-V5 antibody (right). WT, wild type. CA, catalytic mutant. Mock, vector control.

described (Loffredo et al., 2020). The cytotoxicity of GRL-0496 was measured by the CellTiter-Glo luminescent cell viability assay (Promega, Wisconsin, USA, #G7570). To evaluate the inhibitory effect of GRL-0496 against SARS-CoV-2 3CLpro, the HEK 293T cells were transfected with 150 ng pGlo-30F-VRLQS, 10 ng pRL-TK and 150 ng of protease expression plasmid. At 14–16 h post-transfection, cells were treated with various concentrations of GRL-0496 (0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 μ M) for 6 h. The cells were lysed and used for luciferase activity assay as described above.

Generation of rabbit polyclonal antiserum to 3CLpro. This

antiserum was generated in 2003 using previously published procedures (Harcourt et al., 2004; Kanjanahaluethai and Baker, 2000; Schiller et al., 1998). Briefly, the 3CLpro region from SARS-CoV was generated by reverse transcription-PCR from viral RNA and cloned in-frame with glutathione S-transferase (GST) in the pGEX-5X-1 vector (Guan and Dixon, 1991) to obtain a GST-3CLpro fusion expression construct. The fusion protein was induced by the addition of isopropyl B-D-thiogalactopyranoside (IPTG) for 2 h, was isolated using affinity chromatography over glutathione-sepharose column, and injected into rabbits for the generation of polyclonal antibodies. The specificity of the antiserum

was evaluated using immunofluorescence assays with virus-infected cells.

Detecting 3CLpro from SARS-CoV-2 infected cells. The immunofluorescence assay was performed as previously described (Loffredo et al., 2020). Briefly, the infected cells were fixed at 12 h post-infection, permeabilized in PBS containing 0.1% Triton X-100 for 15 min at room temperature, incubated with a blocking buffer (PBS containing 0.1% Triton X-100, 10% normal goat serum, 1% bovine serum albumin) for 1 h at room temperature. To test the anti-3CLpro serum, we co-stained the infected cells with a mouse anti-dsRNA antibody clone rJ2 (Millipore Sigma, #MABE1134; 1:1000 dilution) and the rabbit anti-3CLpro serum (1:1000 dilution) overnight at 4 °C. The next day, the cells were stained with Alexa 568-conjugated anti-rabbit antibody (1:1000 dilution) and Alexa 488-conjugated anti-mouse antibody (1:1000 dilution) for 1 h and counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Images were captured using Olympus IX71 microscope at 60× magnification and analyzed with imaging software system of Imaris x64 8.0.2.

Western blot detection of SARS-CoV-2 3CLpro/nsp5 from infected cells. Caco-2 cells engineered to express ACE2 and TMPRSS2 (Caco-2 ACET2/TMPRSS2) were infected with SARS-CoV-2 at a multiplicity of infection of 5 and harvested at 8, 12, and 24 h post-infection. Uninfected cells harvested in parallel served as controls. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane followed by blocking in 5% bovine serum albumin in TBST buffer for 1 h at room temperature. The membrane was incubated for overnight at 4 °C with the rabbit anti-3CLpro serum diluted 1:1000 in the blocking buffer. The next day, the membrane was washed and incubated with 1:5000 dilution of IRDye 800CW goat anti-rabbit secondary antibody (LI-COR, #926–32211) in the dark for 1 h at room temperature. Protein bands were visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

Western blot detection of 3CLpro cleavage products expressed from plasmids. HEK 293T/17 cells were plated at a density of 7.0×10^5 cells in 35-mm dishes (Corning, #430165) and transfected with 1 µg of the indicated expression plasmids using the TransIT-LT1 transfection reagent (Mirus). At 24 h post-transfection, cells were lysed with 200 µl of lysis buffer A, containing 4% SDS, 3% dithiothreitol (DTT), and 0.065 M Tris, pH 6.8. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% methanol) for 30 min at 25 V. The membrane was blocked using 5% dried skim milk in TBST buffer (0.9% NaCl, 10 mM Tris-HCl, pH 7.6, 0.1% Tween 20) overnight at 4 °C. The next day, the membrane was incubated with a 1:5000 dilution of mouse monoclonal anti-V5 antibody (Invitrogen) followed by incubation with a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (SouthernBiotech). Secondary antibody was detected using Western Lighting Plus chemiluminescence reagent (PerkinElmer) and visualized using a FluorChem E imager. The same membrane was used to test the anti-SARS-CoV 3CLpro antiserum. For this, we stripped the membrane by incubating with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 0.8% β-mercaptoethanol) at 55 °C for 30 min and blocked it once again in the blocking buffer. The membrane was then incubated with a 1:3000 dilution of the rabbit anti-3CLpro serum, followed by incubation with a 1:5000 dilution of HRP-conjugated donkey anti-rabbit IgG antibody (SouthernBiotech). The protein bands were detected using a Western Lighting Plus chemiluminescence reagent and visualized using a FluorChem E imager.

Statistical analysis. All statistical data analyses and plots were performed in Graphpad Prism 8 and verified SAS version 9.4 software; p-values of less than 0.05 were considered statistically significant. Dose response curves were fitted for the independent triplicate firefly luciferase/renilla luciferase ratio measurements for each protein using the NLIN (nonlinear) SAS procedure and the four-parameter Hill equation, which includes the IC₅₀ parameter (Seber and Wild, 1989). Likelihood-ratio F tests demonstrated that each of these IC₅₀s differed significantly with p-values <0.0001 for both comparisons with

MERS-CoV 3CLpro and p = 0.0122 comparing the IC₅₀s of SARS-CoV 3CLpro and SARS-CoV-2 3CLpro.

CRedit authorship contribution statement

Amornrat O'Brien: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Da-Yuan Chen:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Matthew Hackbart:** Investigation, Methodology, Writing - review & editing. **Brianna J. Close:** Investigation, Methodology, Writing - review & editing. **Timothy E. O'Brien:** Formal analysis, Writing - review & editing. **Mohsan Saeed:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Susan C. Baker:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virol.2021.01.010>.

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