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Development of a Complementation Assay to Monitor Pan-Coronavirus 3C-like Protease Activity

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Abstract

Coronaviruses pose a global pandemic threat, making development of a pan-coronavirus inhibitor crucial for preparedness and containment in the event of a new coronavirus outbreak. The 3C-like protease (3CL^{Pro}) is a key target for antiviral development, as it is essential for viral replication and conserved across human coronaviruses. We previously developed an assay to monitor SARS-CoV-2 3CL^{Pro} activity in cells. This assay uses a single vector that coexpresses the 3CL^{Pro} enzyme and the reporter, which consists of two luciferase fragments linked by a 3CL^{Pro} cleavage site. Cleavage of this site by 3CL^{Pro} decreases luciferase activity, whereas inhibition of 3CL^{Pro} increases the luciferase activity. Here, we adapted this assay to examine 3CL^{Pro} activity from six other human coronaviruses: SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, HCoV-OC43, and HCoV-HKU1. We further determined the effects of different cleavage sites to improve the signal-to-background ratio. The Nsp4-Nsp5 site and super-active substrate (SAS) resulted in the largest dynamic range for most coronaviruses in our assay. Using the broad-spectrum 3CL^{Pro} inhibitor GC376, we observed increased reporter activity, indicating the assay's efficacy for identifying inhibitors across multiple coronaviruses. The adaptation and improvement of the assay can facilitate the development of inhibitors against 3CL^{Pro} from multiple or novel coronaviruses.

Keywords: coronaviruses; 3CL^{Pro}; luciferase reporter assay; pan-coronavirus inhibitor

1. Introduction

In the past two decades, three distinct coronaviruses with pandemic potential have been introduced into human population by zoonotic transmissions, including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 [1–5]. SARS-CoV and MERS-CoV led to limited spread within the human population, whereas SARS-CoV-2 has resulted in a pandemic with more than 700 million reported cases and over 7 million human fatalities [6]. Currently, seven species of coronaviruses are known to cause infections in humans; they belong to the genus *Alphacoronavirus* (HCoV-229E and HCoV-NL63) and *Betacoronavirus* (HCoV-HKU1, HCoV-OC43, MERS-CoV, SARS-CoV, and SARS-CoV-2). Of these viruses, SARS-CoV and MERS-CoV can cause severe respiratory infections with the potential for high case fatality rates, whereas SARS-CoV-2 exhibits a lower fatality rate with high transmissibility [7,8]. HCoV-229E, HCoV-NL63, HCoV-HKU1, and HCoV-OC43 generally cause mild upper



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respiratory tract infections but can be fatal in immunocompromised individuals [9]. Thus, multiple members of the *Coronaviridae* family are human pathogens; of these, at least three are novel human viruses caused by zoonotic infections, some with catastrophic impacts on a worldwide scale. These findings suggest that coronaviruses are likely to remain a future pandemic threat, and it is imperative to develop antiviral strategies to counter coronavirus replication and contain or mitigate future outbreaks.

Antivirals can reduce the severity of diseases in infected individuals and control the spread of viral infection within the human population. Besides immunomodulators such as monoclonal antibodies, remdesivir (Veklury) and nirmatrelvir/ritonavir (Paxlovid) are two FDA-approved antivirals for the treatment of COVID-19 [10,11]. Remdesivir targets the viral RNA-dependent RNA polymerase, whereas nirmatrelvir targets the 3C-like protease (3CL^{Pro}) of SARS-CoV-2 [10,11]. 3CL^{Pro}, also known as main protease (M^{Pro}) and non-structural protein 5 (Nsp5), is a cysteine protease required for viral replication [12]. 3CL^{Pro} cleaves at least 11 cleavage sites of the coronavirus polyprotein 1a (pp1a) and pp1ab, generating non-structural proteins [12]. 3CL^{Pro} is as an attractive target for antiviral development because its activity is required for coronavirus replication, and its cleavage specificity is distinct from human proteases likely reducing off target effects [13]. Furthermore, the success of nirmatrelvir in preventing COVID-19-related hospitalization and death validates the feasibility of using antivirals targeting 3CL^{Pro} [14]. Additionally, the structure of 3CL^{Pro} active site is highly conserved across different coronaviruses [15,16]; this feature makes it possible to develop small molecules that can inhibit 3CL^{Pro} from multiple coronaviruses.

A critical component of antiviral development is the assay used to monitor the target activity and evaluate the effects of potential compounds against the target. A frequently used approach to monitor SARS-CoV-2 3CL^{Pro} activity is an in vitro fluorescence resonance energy transfer (FRET)-based method [17–20]. This approach uses a dually labeled substrate in which the close proximity of the labels quenches the fluorescence signals. When purified recombinant 3CL^{Pro} is added to the reaction, proteolytic cleavage of the substrate leads to separation of the two labels that increases fluorescent signals [17–20]. Although amenable to high-throughput screening, the in vitro FRET-based assay cannot account for cellular properties of small molecules such as permeability, metabolic rate, and cytotoxicity [17–20]. To overcome this, several cell-based assays have been established, including loss-of-function assays in which reporter activity is reduced by the presence of an inhibitor. The drawback of this type of assay is that both inhibition of target activity and cytotoxicity caused by the compound lead to a positive readout; thus, assays using this strategy often have false-positives [21–24]. An assay has been reported in which inhibition of proteolytic activity leads to the activation of a synthetic transactivator protein that activates the reporter gene [25]. Another cell-based assay depends on the requirement of 3CL^{Pro} cleavage activity to restore viral replication that can be scored [26]. The readout of the last two approaches is indirect, since 3CL^{Pro} cleaves an intermediate which then affects the reporter signal. Such strategies may have experimental complications resulting in false readings. Additionally, many assays require co-transfection of multiple plasmids [27,28], which can increase variability in the system.

We have recently described a cell-based assay to monitor SARS-CoV-2 3CL^{Pro} activity that can be performed in a BSL-2 setting using a single vector [29]. In this assay, the vector delivers both 3CL^{Pro} and a split luciferase reporter substrate containing a 3CL^{Pro} cleavage site. When the reporter is cleaved by 3CL^{Pro}, the reporter signal is diminished. In contrast, when the 3CL^{Pro} activity is inhibited, the reporter maintains its signal. Thus, in this system, the inhibition of the 3CL^{Pro} results in an increase in reporter function. The advantages of the system are that it can easily distinguish between cytotoxicity and inhibitory effect, and the reporter contains the target site, so the readout directly reflects the cleavage of the

reporter, avoiding potential complications of indirect readout systems. Additionally, the usage of a single plasmid simplifies the delivery and makes the assay highly adaptable.

In the current report, we have expanded our previously described work to monitor 3CL^{Pro} activity from multiple human coronaviruses including HCoV-229E, HCoV-NL63, HCoV-HKU1, HCoV-OC43, MERS-CoV, and SARS-CoV. The 3CL^{Pro} targets multiple cleavage sites that are not conserved among these human coronaviruses. To determine an optimal reporter sequence, we have also evaluated different cleavage substrates embedded within the reporter protein including two naturally occurring cleavage sites from the autologous virus, and a cleavage site identified by substrate specificity profiling [30]. We have found that our assay can detect activity from all seven 3CL^{Pro} enzymes; furthermore, the cleavage site embedded within the reporter affects the dynamic ranges of the assay. Together, these studies establish a cell-culture-based assay to monitor the 3CL^{Pro} activity for seven human coronaviruses. The characterization of this assay provides the ability to monitor 3CL^{Pro} activities across multiple human coronaviruses, demonstrates the flexibility of this assay, and paves the way to identify pan-coronavirus antivirals. The identification of a substrate that can be efficiently cleaved by enzymes from multiple human coronaviruses simplifies steps required to adapt this assay to monitor 3CL^{Pro} activity of a novel coronavirus. These studies improve our readiness for potential future coronavirus pandemics.

2. Materials and Methods

2.1. Plasmids

Vectors used in this study were derived from a previously described lentiviral vector (S-L-GFP) [29]. Briefly, this vector encodes a codon-optimized 3CL^{Pro} from SARS-CoV-2, which was fused with a self-cleavable peptide, P2A from porcine teschovirus-1 (GSGAT-NFSLKQAGDVEENPGP), followed by the Small (S) and the Large (L) fragment of a split NanoLuc luciferase reporter gene, and a green fluorescent protein (GFP) gene. The 3CL^{Pro} cleavage site between SARS-CoV-2 Nsp4 and Nsp5 (referred to as Nsp4-Nsp5 site) separates the S and L fragments of NanoLuc; additionally, two flexible linkers flank the Nsp4-Nsp5 cleavage site, each with the GGGSGGGSGGGS sequence.

To generate vectors encoding 3CL^{Pro} and cleavage site between Nsp14 and Nsp15 (referred to as Nsp14-Nsp15 site) of other CoVs, gBlocks were synthesized (Integrated DNA Technologies, Coralville, IA, USA) to contain codon-optimized 3CL^{Pro} from SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, HCoV-HKU1, or HCoV-OC43, followed by P2A, Small BiT (S) of NanoLuc, and a stretch of 12 amino acids encompassing the Nsp14-Nsp15 cleavage site from the corresponding CoV with flanking EcoRI and XbaI sites at the 5' and 3' ends, respectively. The gBlocks were then digested with EcoRI and XbaI and inserted into the previously described SARS-CoV-2 3CL^{Pro} vector S-L-GFP digested with EcoRI and XbaI. To generate a vector with the SARS-CoV-2 3CL^{Pro} and the Nsp14-Nsp15 site, a gBlock containing the SARS-CoV-2 Nsp14-Nsp15 site with a portion of the P2A sequence at the 5' end and a portion of the linker sequence at the 3' end was synthesized. This fragment was used to replace the corresponding fragment in the S-L-GFP vector by NsiI and XbaI enzyme digestion and ligation.

Similarly, the Nsp4-Nsp5 site with flanking restriction sites was synthesized (Integrated DNA Technologies) and used to replace the Nsp14-Nsp15 site in existing vectors. The Nsp4-Nsp5 sites of SARS-CoV and SARS-CoV-2 share the same amino acid sequence. To generate a vector with SARS-CoV 3CL^{Pro} and the Nsp4-Nsp5 cleavage site, the fragment containing the Nsp4-Nsp5 cleavage site from the SARS-CoV-2 3CL^{Pro} vector was isolated and inserted into the SARS-CoV 3CL^{Pro}-containing vector to replace the Nsp14-Nsp15 site. Similarly, gBlocks containing the site identified through substrate specificity profiling, termed super-active substrate (SAS) [30] and MERS-CoV Nsp5-Nsp6 were created and

inserted into the MERS-CoV 3CL^{pro} vector. Finally, the SAS site from the MERS-CoV 3CL^{pro} vector containing the SAS site was extracted and inserted into the remaining CoV vectors.

To generate control vectors encoding inactivated 3CL^{pro} (mut3CL^{pro}), the conserved catalytic cysteine residue of each 3CL^{pro} was substituted with alanine by site-directed mutagenesis of the plasmid. For SARS-CoV-2, SARS-CoV, HCoV-HKU1, and HCoV-OC43, the cysteine residue at the 145th position of 3CL^{pro} was changed to alanine (C145A). The corresponding active site mutation for MERS-CoV 3CL^{pro} is C148A, and for HCoV-NL63 and HCoV-229E 3CL^{pro} are C144A.

2.2. Transfections and Luciferase Assays

Human 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. For transfection, 1×10^4 293T cells in 0.1 mL medium were seeded into each well of a 96-well plate. Cells were transfected 24 h later with 100 ng of lentiviral vector plasmid using TransIT-LT1 reagent (Mirus Bio; Madison, WI, USA). At 4 h post-transfection, 1.1 µL of dimethyl sulfoxide (DMSO) or GC376 in DMSO (Selleck Chemicals; Houston, TX, USA) was added to each well. Luciferase activity was determined 24 h post-transfection using the Nano-Glo Live Cell Assay System (Promega; Madison, WI, USA), following the manufacturer's instructions. Briefly, after removing the culture medium, 100 µL Opti-Mem reduced serum medium (Life Technologies; Carlsbad, CA, USA), 23.75 µL Nano-Glo dilution buffer (Promega; Madison, WI, USA), and 1.25 µL Nano-Glo live cell-substrate was added to each well. After mixing on an orbital shaker for 15 s, the plate was read immediately using a SpectraMax iD3 (Molecular Devices; San Jose, CA, USA) with an integration time of 400 ms. Luciferase values obtained were normalized to those of wildtype controls and averaged from at least three independent experiments. For the dose-response analysis, luciferase values were normalized to the DMSO and 100 µM GC376 treated samples, which were set to 0 and 100%, respectively.

2.3. Western Blotting

To measure luciferase reporter activity and reporter cleavage in the same transfection experiment, 293T cells were plated into 6-well plates at a density of 3.3×10^5 with 2 mL medium per well and 24 h later transfected with 0.6 µg of plasmid and 1 µL TransIT-LT1 reagent (Mirus Bio). At 4 h post-transfection, cells were split into a solid white 96-well plate ($\sim 1 \times 10^4$ cells per well) and a 24-well plate ($\sim 6 \times 10^4$ cells per well), and either DMSO or 100 µM GC376 was added. At 30 h post-transfection, the luciferase activity was measured using cells in the 96-well plate whereas western blotting was performed using cells from the 24-well plate. Cells in the 24-well plates were lysed using CellLytic M solution (Sigma-Aldrich; St. Louis, MO, USA) containing cOmplete EDTA-free protease inhibitor cocktail tablets (Roche; Basel, Switzerland). Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C (Eppendorf 5425R centrifuge), and 75 µL of supernatants were mixed with 25 µL of 4× Laemmli sample buffer (Bio-Rad; Hercules, CA, USA) containing 10% beta-mercaptoethanol. Following denaturation at 99 °C for 5 min, samples were loaded onto a 4–20% Criterion TGX precast gel (Bio-Rad). After transfer to PVDF membranes, blots were probed with 1:5000 rabbit anti-GFP antibodies (Invitrogen; Carlsbad, CA, USA) and goat anti-rabbit IgG (IRDye 800RD, LI-COR, Lincoln, NE, USA) secondary antibodies at 1:10,000 dilutions. Blots were also sequentially probed with mouse anti-P2A (Novus Biologicals, Littleton, CO, USA) and mouse anti-HSP90 (Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies, followed by goat anti-mouse IgG (IRDye 680RD, LI-COR) as the secondary antibody. Western blots were imaged using the Odyssey CLx infrared imaging system (LI-COR).

2.4. Cell Viability Analysis

Cell viability was assessed using the CellTiter-Glo 2.0 Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, 293T cells were seeded, transfected, and treated with either DMSO or GC376 as described above. Cell viability was measured 24 h post-transfection by adding 100 µL of CellTiter-Glo 2.0 reagent to each well. Plates were mixed for 2 min on an orbital shaker, and luminescence was measured 10 min later using a SpectraMax iD3 plate reader (Molecular Devices). Luciferase activity from GC376-treated cells was normalized to that of DMSO-treated cells, which was set to 100%.

2.5. Statistical Analysis

Normalized data from three biological replicates were analyzed using a mixed-effects model, with treatment as a fixed effect and replicate as a random effect. Tukey's multiple comparison test was applied for pairwise comparisons. Statistical analysis was performed in GraphPad Prism v10.6.1 (GraphPad Software, San Diego, CA, USA).

Dose-response curves were generated by plotting agonist concentration versus normalized response using a variable-slope nonlinear regression model. For each viral 3CL^{Pro} and cleavage substrate, curves were fitted using nonlinear regression to estimate inhibitory responses.

3. Results

3.1. Development of Reporter Vectors to Measure 3CL^{Pro} Activities of Multiple Coronaviruses

To monitor 3CL^{Pro} activities of multiple human coronaviruses, we expanded the previously described NanoLuc complementation assay [29,31]; in this assay, the NanoLuc luciferase is divided into two fragments, S and L, that are connected by a linker containing a 3CL^{Pro} cleavage site (Figure 1A). Complementation of the S and L fragments forms a functional luciferase protein with detectable activity. Cleavage of the linker by 3CL^{Pro} results in dissociation of the S and L fragments and the loss of luciferase activity. Thus, inhibiting the target site cleavage by 3CL^{Pro} results in an increase in luciferase activity (Figure 1A). We have previously constructed a lentiviral vector that expresses both the SARS-CoV-2 3CL^{Pro} and the NanoLuc reporter gene (Figure 1B). The 3CL^{Pro} is expressed by an internal promoter followed by a self-cleaving peptide (P2A) that allows the expression of the split NanoLuc. The S and L fragments of NanoLuc are separated by the SARS-CoV-2 3CL^{Pro} Nsp4–Nsp5 cleavage site. A GFP gene is fused to the C-terminus of the L fragment to facilitate the monitoring of transfection efficiency and 3CL^{Pro} cleavage of the NanoLuc using western blot analysis.

To expand this assay, we replaced the SARS-CoV-2 3CL^{Pro} with 3CL^{Pro} from SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, HCoV-OC43, and HCoV-HKU1. Coronavirus 3CL^{Pro} proteins cleave their polyproteins at multiple sites and these sites have different sequences. Additionally, the sequences of the cleavage sites are not necessarily conserved among different viruses, making it essential to match the enzyme with the autologous cleavage sites. We first examined the Nsp14–Nsp15 cleavage site, which is the most conserved cleavage site among these seven coronaviruses (alignment shown in Figure 1C). We reasoned that the high conservation of the cleavage site could facilitate the identification of an equivalent site in a novel virus, making the assay easier to adapt. As a negative control, we generated a nonfunctional 3CL^{Pro} active site mutant (mut3CL^{Pro}) for each construct that contained an alanine in place of the catalytic cysteine residue.

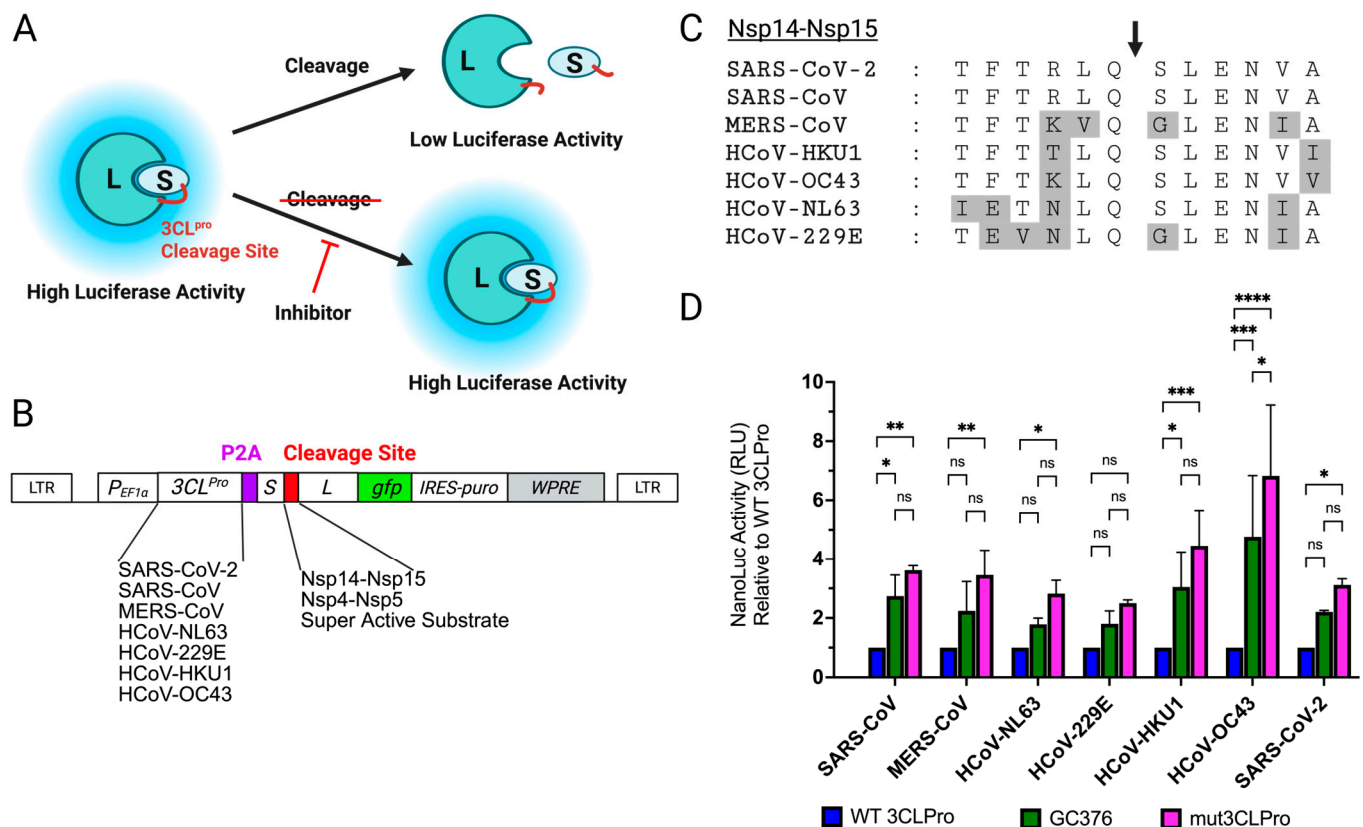


Figure 1. Construction and testing of vectors expressing 3CL^{Pro} and NanoLuc reporter containing autologous Nsp14-Nsp15 cleavage site. **(A)** Using a split NanoLuc reporter to detect 3CL^{Pro} activity. The Small (S) and Large (L) fragment of NanoLuc is connected by a linker containing a 3CL^{Pro} cleavage site. Cleavage by 3CL^{Pro} leads to dissociation of the two fragments and a loss of luciferase activity. Inhibitors or mutation of the active site of 3CL^{Pro} prevent the 3CL^{Pro} cleavage, maintaining the association of L and S fragments and luciferase activity. **(B)** General structure of the lentiviral vector expressing 3CL^{Pro} and reporter. The S and L fragments of NanoLuc are separated by a 3CL^{Pro} cleavage site. Sites tested include Nsp14-Nsp15 sites, Nsp4-Nsp5 sites, and a previously reported super-active substrate [29]. LTR, long terminal repeat; P_{EF1α}, EF1α promoter; P2A, self-cleavable peptide from porcine teschovirus-1; GFP, green fluorescent protein; IRES, internal ribosome entry site; puro, puromycin resistance gene; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. **(C)** Amino acid sequence alignments of Nsp14-Nsp15 cleavage sites from seven human coronaviruses. Amino acids that differ from those of SARS-CoV-2 are shaded in gray. The site of 3CL^{Pro} cleavage is depicted by the arrow. **(D)** NanoLuc activity detected in the system. WT 3CL^{Pro} and GC376 refer to results generated from cells transfected with vectors expressing WT 3CL^{Pro} treated with DMSO and GC376, respectively. Mut3CLPro refers to results generated from cells transfected with vectors expressing 3CL^{Pro} containing a cysteine-to-alanine catalytic site mutation. NanoLuc activities were normalized to those obtained with the autologous WT 3CL^{Pro}. Data from three independent experiments are shown as mean ± SD. The blue bars indicate samples transfected with vectors expressing WT 3CL^{Pro}, the green bars indicate WT 3CL^{Pro} samples treated with GC376, and the magenta bars indicate samples transfected with vectors expressing the catalytically inactive mut3CL^{Pro}. Statistical significance is indicated as follows: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, ns—not significant.

To examine vectors containing autologous Nsp14-Nsp15 cleavage sites, each plasmid was transfected into 293T cells, and the luciferase activities were measured 24 h after transfection. Results from three sets of experiments are summarized in Figure 1D. The mut3CL^{Pro} does not have protease activity and cannot cleave the reporter; thus, the mut3CL^{Pro} samples are expected to generate higher luciferase activities compared with

samples that express functional 3CL^{Pro}. In all seven vectors, the mut3CL^{Pro} luciferase activities (magenta bars) are higher than those from the WT 3CL^{Pro} (blue bars). The low luciferase activity for WT indicates that the 3CL^{Pro} in each vector is expressed, and its activity is detected using the Nsp14-Nsp15 cleavage site. However, in all cases, the differences between activities detected in WT 3CL^{Pro} and mut3CL^{Pro} samples, which indicate the dynamic ranges of the assays, are modest; they ranged from 2.5-fold (HCoV-229E) to 6.8-fold (HCoV-OC43). To illustrate that this assay can detect partial inhibition of 3CL^{Pro} activity, such as that from a potential small-molecule inhibitor, we have also added controls in which cells expressing WT 3CL^{Pro} were treated with GC376. GC376 is a broad-spectrum protease inhibitor originally developed to inhibit the 3CL^{Pro} of feline coronavirus (FCoV) and can also inhibit 3CL^{Pro} from SARS-CoV-2 and other CoVs [32–34]. We have previously shown that treating cells with 100 µM GC376 partially inhibit SARS-CoV-2 3CL^{Pro} activity and does not induce cytotoxicity [29]. Other reports also showed that GC376 can be used at concentrations up to 100 µM without causing significant cytotoxicity [35,36]. Cells were treated with a final concentration of 100 µM for 20 h prior to performing the luciferase assay [29]. In all experimental groups, the luciferase values from GC376-treated samples were between those from WT and mut3CL^{Pro}, suggesting that GC376 partially inhibits various 3CL^{Pro} enzymes at this treatment condition. We also performed a cell viability assay and found that treatment with 100 µM GC376 did not cause detectable reduction in cell viability (Supplementary Figure S1). The detection of the 3CL^{Pro} activity from each vector was encouraging; however, the modest dynamic range of 3CL^{Pro} activity detected indicated further improvement was needed.

3.2. Examining the Effects of Cleavage Sites on the Dynamic Range of the Assay

In our previous study, we observed a 10-fold dynamic range using SARS-CoV-2 3CL^{Pro} and the autologous Nsp4-Nsp5 cleavage site [29]. These results are in contrast to the <4-fold dynamic range when the Nsp14-Nsp15 site is used (Figure 1D) and suggest that the cleavage site usage impacts the dynamic range of the assay. This is consistent with previous reports demonstrating that 3CL^{Pro} can display different activity when acting on various cleavage sites. For example, the SARS-CoV and SARS-CoV-2 3CL^{Pro} are more active in cleaving the Nsp4-Nsp5 site compared to other sites [18,37,38], whereas the MERS-CoV 3CL^{Pro} cleaves the Nsp5-Nsp6 site more efficiently than the Nsp4-Nsp5 site [39]. To improve the reporter system, we modified our constructs so that each vector expressed a 3CL^{Pro} and the autologous Nsp4-Nsp5 cleavage site between the S and the L fragments. The alignment of the Nsp4-Nsp5 cleavage sites of seven coronaviruses is shown in Figure 2A. Additionally, we generated a vector that expressed a MERS-CoV 3CL^{Pro} and the corresponding Nsp5-Nsp6 cleavage site within the NanoLuc. As a control, we generated a plasmid mirroring each vector but encoding catalytically inactive 3CL^{Pro}.

We then examined whether replacing protease cleavage sites improved the dynamic range of 3CL^{Pro} cleavage; results from three independent experiments are summarized in Figure 2B. We observed that, compared to those from vectors with the Nsp14-Nsp15 site, the dynamic ranges improved in some of the vectors such as SARS-CoV (from 3.6-fold in Figure 1D to 12.1-fold in Figure 2B), HCoV-NL63 (from 2.8-fold in Figure 1D to 8.7-fold in Figure 2B) and HCoV-229E (from 2.5-fold in Figure 1D to 5.7-fold in Figure 2B). However, such improvement is not observed in HCoV-HKU1, or HCoV-OC43. For MERS-CoV, the dynamic ranges remained modest when using Nsp4-Nsp5 or Nsp5-Nsp6 cleavage sites (5.1-fold and 6.5-fold, respectively). GC376 treatment resulted in detectable inhibition of 3CL^{Pro} activity from all seven enzymes, as indicated by increased luciferase activity in the reporter assay. These results illustrated that the dynamic range of the detection can be

improved by optimizing the cleavage site between S and L fragments. However, the best cleavage site for each virus may vary and require optimization.

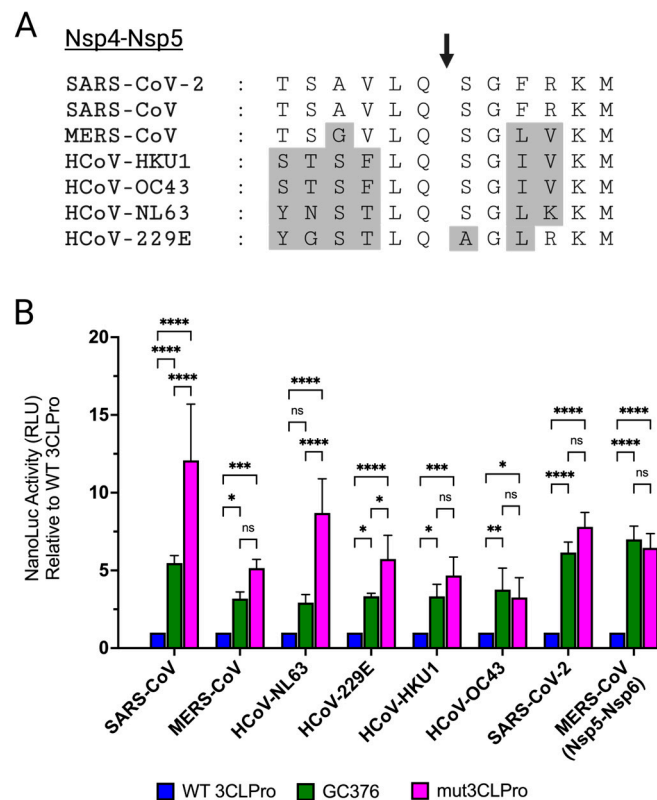


Figure 2. NanoLuc reporters with autologous Nsp4-Nsp5 or Nsp5-Nsp6 cleavage sites. (A) Amino acid sequence alignments of Nsp4-Nsp5 cleavage sites of seven coronaviruses. Amino acids that differ from those of SARS-CoV-2 are shaded in gray. The site of 3CL^{Pro} cleavage is depicted by the arrow. (B) NanoLuc activities of cells transfected with vectors and the inhibition by GC376 treatment. NanoLuc activities are normalized to WT 3CL^{Pro}. Data represent three independent experiments shown in mean $\pm$ SD. Abbreviations and color scheme are the same as Figure 1. Blue bars, samples with WT 3CL^{Pro}; green bars, samples with WT 3CL^{Pro} treated with GC376; magenta bars, samples with catalytically inactive mut3CL^{Pro}. Statistical significance is indicated as follows: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, ns—not significant.

3.3. Utilizing an Artificial Cleavage Site to Monitor the Activities of Multiple HCoV 3CL^{Pro}

One of our main goals is to provide an assay that can be rapidly adapted to test compounds for novel emerging coronaviruses. The requirement to identify a suitable autologous 3CL^{Pro} cleavage site is a hindrance to the rapid deployment of the assay. To overcome this issue, we tested whether one cleavage site can be used to detect 3CL^{Pro} activities of multiple viruses. For this purpose, we examined a previously described super-active substrate (SAS) that was identified through substrate library screening and shown to be cleaved by SARS-CoV, HCoV-NL63, and HCoV-OC43 3CL^{Pro} [30]. We replaced the 3CL^{Pro} cleavage site sequences in our vectors with the sequence encoding the SAS site (VARLQ ↓ SGF; arrow indicated the cleavage site). As a control, we generated a plasmid identical to each of the test vectors except encoding the catalytically inactivated 3CL^{Pro} enzyme described above.

We measured the luciferase activities produced from cells transfected with these vectors and found that in all seven HCoV 3CL^{Pro} samples, the luciferase activities were much higher in the mut3CL^{Pro} samples compared to those from the WT 3CL^{Pro} (Figure 3B), indicating all tested 3CL^{Pro} can use the SAS target site. Furthermore, the dynamic range

of these constructs were equivalent or better than those using an authentic autologous cleavage site (Figures 1D and 2B). For example, the dynamic range for SARS-CoV-2 3CL^{Pro} was improved to ~20-fold when using the SAS site compared to ~8-fold from using the Nsp4-Nsp5 cleavage site (Figures 2B and 3B), which is the most efficient cleavage site among the 11 sites in SARS-CoV-2 [18]. Similarly, HCoV-OC43 vectors with the SAS site illustrated dynamic ranges ~10-fold compared to ~7-fold and ~3-fold when using the Nsp14-Nsp15 site and Nsp4-Nsp5 site, respectively (Figure 3B vs. Figures 1D and 2B). For SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, and HCoV-HKU1, the dynamic ranges detected using the SAS cleavage site were similar to those using Nsp4-Nsp5 site (Figure 3B vs. Figures 1D and 2B).

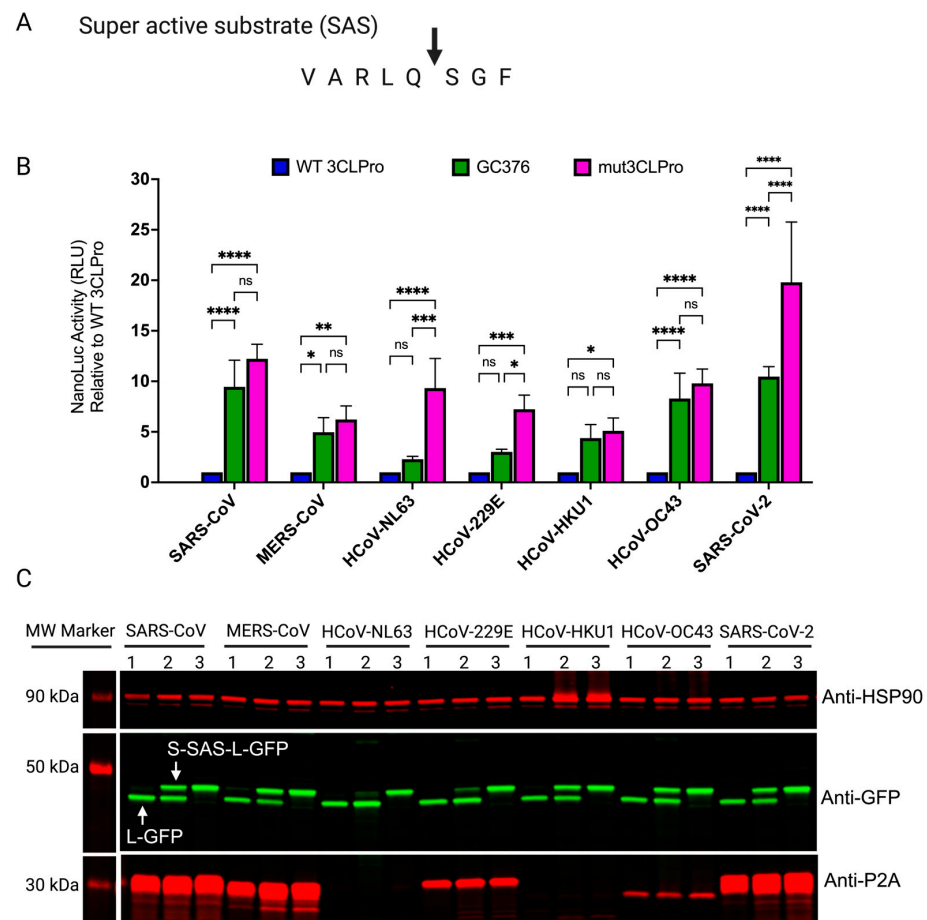


Figure 3. Dynamic range of the assay using an artificial 3CL^{Pro} cleavage site detected by NanoLuc activity and target site cleavage detected by western blot. **(A)** Amino acid sequence of the super-active substrate [30]. The site of 3CL^{Pro} cleavage is depicted by the arrow. **(B)** NanoLuc activity when using the artificial SAS target site. Results are normalized to WT 3CL^{Pro}. Data represent three independent experiments shown as the mean $\pm$ SD; abbreviations and color scheme are the same as Figure 1. Blue bars, samples with WT 3CL^{Pro}; green bars, samples with WT 3CL^{Pro} treated with GC376; magenta bars, samples with catalytically inactive mut3CL^{Pro}. **(C)** Cleavage of the SAS target site within the reporter detected by western blotting. HEK293T cells were transfected with 3CL^{Pro} WT (lanes marked 1 and 2) or catalytically inactive mutants (lanes marked 3) and treated with DMSO (lanes marked 1) or the 3CL^{Pro} inhibitor GC376 (lanes marked as 2). Total cell lysates were subjected to western blotting. NanoLuc reporters (cleaved and uncleaved) were detected using an anti-GFP antibody (green, middle panel). Upper arrow denotes the uncleaved functional reporter (S-SAS-L-GFP) and lower arrow denotes cleaved reporter (L-GFP). 3CL^{Pro} was detected using anti-P2A antibodies (red, bottom panel). Statistical significance is indicated as follows: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, ns—not significant.

Next, we examined the lysates of transfected cells by western blotting analyses to determine the expression of the reporter gene and whether the luciferase activity detected in our assay correlates with the cleavage of the SAS site between the S and L fragments. In our vectors, the NanoLuc L fragment is fused to GFP (Figure 1B), allowing us to use an anti-GFP antibody to examine expression and cleavage of the reporter by western blotting (Figure 3C). The intact reporter is ~49 kDa and contains the S fragment, linker including the SAS site, L fragment, and GFP (S-SAS-L-GFP). If the SAS site is cleaved, the anti-GFP antibody will detect the L-GFP protein, which is ~46 kDa. A representative western blot is shown in Figure 3C; for each 3CL^{Pro} examined, the WT 3CL^{Pro} is shown in lane 1, the GC376 treated WT 3CL^{Pro} is shown in lane 2, and the catalytic site mutant 3CL^{Pro} is shown in lane 3. For example, in SARS-CoV samples, a band corresponding to L-GFP is detected in WT 3CL^{Pro} whereas a band corresponding to the entire reporter (S-SAS-L-GFP) is detected in mut3CL^{Pro} (Figure 3C, lane 1 and lane 3, respectively). These results are consistent with the ability of WT 3CL^{Pro}, but not the mut3CL^{Pro}, to cleave the SAS site within the reporter and the lower luciferase activity detected in the WT 3CL^{Pro} samples compared to that from the mut3CL^{Pro} (Figure 3B). Two bands corresponding to the intact reporter and the L-GFP are detected in GC376 treated sample (Figure 3C, lane 2), consistent with incomplete inhibition of the 3CL^{Pro} by the treatment condition and the observed intermediate luciferase activity. Similar patterns are observed with other 3CL^{Pro} samples as well; some of the GC376 treated samples have different ratios of upper and lower reporter bands, consistent with our luciferase activity results indicating that GC376 treatment have different inhibitory effects on various 3CL^{Pro} (Figure 3B; quantification of the western blots is shown in Supplemental Figure S2). Thus, these western blot results demonstrate that the differential luciferase activities detected in our system are from cleavage of the target site within the reporter. Additionally, these results also showed that the reporter is expressed at similar level in all seven vectors regardless of the identity of the 3CL^{Pro}. In our vector, the P2A peptide is fused to the C-terminus of 3CL^{Pro}. With this feature, we have used an antibody against P2A to detect 3CL^{Pro} proteins from various viruses. In some samples, such as those from SARS-CoV, MERS, and SARS-CoV-2, we found strong bands corresponding to 3CL^{Pro}-P2A, and some samples had bands of moderate intensities, such as those from HCoV-229E and HCoV-OC43. Detection of the HCoV-NL63 and HCoV-HKU1 3CL^{Pro} expression using this approach yielded little signals although we consistently detected lower luciferase activities in WT samples compared to mut3CL^{Pro} samples (Figure 3B), indicating these enzymes were expressed. Because an anti-P2A antibody was used, cleavage or masking of the P2A peptide can lead to reduced or inability to detect the 3CL^{Pro} protein in the western blot, thereby explaining the positive enzyme activity in luciferase assay but lacking a corresponding band in the western blot.

3.4. Dose–Response Analysis of GC376 Against Coronavirus Proteases Using Autologues and Artificial Cleavage Sites Substrates

To determine whether this assay can respond to lower amounts of inhibitor, we have transfected cells with vectors and then treated cells with DMSO or increasing concentrations of GC376 (0.1 µM, 0.4 µM, 1.6 µM, 6.4 µM, 25 µM, and 100 µM). We observed increased luciferase activities at higher GC376 concentrations compared to those with lower concentration of compounds in all seven of the 3CL^{Pro} enzymes tested. Additionally, this feature is observed in vectors with reporters containing the SAS or the Nsp4–Nsp5 cleavage site (Supplemental Figure S3). These results indicate that this assay respond to different doses of GC376 and provide support for its usage to evaluate compounds for their ability to inhibit coronavirus 3CL^{Pro} enzymes.

4. Discussion

Zoonotic transmissions can introduce pathogens into the human population with pandemic potential. Three different zoonotic coronaviruses capable of human-to-human spread have been documented in the past two decades. These events illustrate the high propensity for coronavirus zoonotic events and their potential threat to global health. Antiviral therapy is an effective tool to reduce the morbidity and mortality of diseases. Inhibition of the SARS-CoV-2 3CL^{pro} is a proven effective antiviral strategy [39]. Although coronaviruses have a high genetic diversity, all known coronaviruses encode a 3CL^{pro} that is required for viral replication. To identify pan-coronavirus antivirals and to prepare for possible emerging pathogens, we have expanded our previously described SARS-CoV-2 3CL^{pro} assay to monitor the activities of 3CL^{pro} from other human coronaviruses. Here, we have demonstrated that the 3CL^{pro} activities can be monitored using a single vector, without the requirement to establish a stable cell line facilitating rapid implementation. Furthermore, we have adapted a SAS cleavage site that can be used by all of the tested 3CL^{pro}. This modification simplifies the steps required to adapt the assay for novel 3CL^{pro} testing, making it possible to insert a coronavirus 3CL^{pro}, transfect cells, and monitor luciferase activity, in a plug-and-use manner. The high flexibility and adaptability provide this system with advantages over other recently reported systems (see below).

Several factors can affect the dynamic range of the assay described in our report: the expression of the 3CL^{pro}, the cleavage of the reporter, and the expression of the reporter. When we used antibody against P2A to detect various 3CL^{pro} proteins, we observed different intensities of corresponding bands with little signals of 3CL^{pro} proteins in samples from HCoV-NL63 and HCoV-HKU1 using the P2A antibody (Figure 3C). It is possible that such variation is caused by the cleavage or masking of the P2A peptide. However, in all of the WT samples, most of the reporter proteins (S-SAS-L-GFP) were cleaved to generate a smaller protein (L-GFP) in western blots (Figure 3C) and cells lysates generated lower luciferase activities (Figure 3B) compared to samples from mut3CL^{pro}, indicating active 3CL^{pro} expression. The translation of the reporter protein (S-SAS-L-GFP) was facilitated by the P2A peptide and appears to be stable across various constructs as shown by the western blot. This is also consistent with our observation that the raw luciferase values detected in vectors expressing various mut3CL^{pro} were within two-fold of one another (Supplementary File). We found that one of the largest factors that affects the dynamic range is the cleavage site used within the reporter protein. We tested multiple sites, including the most conserved cleavage site of all seven coronaviruses, Nsp14-Nsp15, as we hypothesized it would serve as a promising substrate for initiating inhibitor screening of a novel coronavirus 3CL^{pro}. However, the Nsp14-Nsp15 cleavage site exhibited a less than 5-fold dynamic range for all tested 3CL^{pro} enzymes, except for the HCoV-OC43 3CL^{pro}, which exhibited a 6.8-fold dynamic range. We also examined the Nsp4-Nsp5 site, which has been shown to be cleaved efficiently by SARS-CoV and SARS-CoV-2 3CL^{pro} enzymes [18,37], and the Nsp5-Nsp6 cleavage site of MERS-CoV, which was reported to be most efficiently cleaved by MERS 3CL^{pro} among all 11 sites [38]. In agreement with previous reports, Nsp4-Nsp5 sites showed a larger dynamic range for SARS-CoV and SARS-CoV-2 (12-fold and 8-fold, respectively). In addition, the dynamic range also improved for MERS-CoV, HCoV-229E, and HCoV-NL63. Thus, the Nsp4-Nsp5 site appeared to be a good substrate for 6 of the 7 coronaviruses tested; the only exception is HCoV-OC43, which exhibited a higher dynamic range with the Nsp14-Nsp15 site compared to that of the Nsp4-Nsp5 site (6.8-fold vs. 3.3-fold respectively; *p* value = 0.0046, mixed-effects analysis). To identify a substrate that can be efficiently cleaved by 3CL^{pro} from most coronaviruses, we tested a previously reported super-active substrate, SAS [30], created by combining the most favorable residues at the cleavage site based on substrate profiles of HCoV-NL63,

HCoV-OC43, SARS-CoV, and infectious bronchitis virus (IBV). However, its specificity for SARS-CoV-2, MERS-CoV, HCoV-229E, and HCoV-HKU1 had not been tested. Our assay showed that SAS exhibited dynamic ranges for most of the coronaviruses that are similar or better than the tested native cleavage sites. Thus, SAS would be the most suitable substrate for the initial screening of pan-coronavirus 3CL^{Pro} inhibitors. In addition, based on the high dynamic range, the SAS sequence can be a useful substrate for SARS-CoV-2 3CL^{Pro} drug screening assays.

Finally, we have shown that treatment with the compound GC376 exhibits inhibitory activity against most of the tested 3CL proteases, although with varying degrees of potency (Figure 3B,C). These results not only demonstrate the feasibility of using our assay for inhibitor screening but also support the possibility of identifying 3CL^{Pro} inhibitors that are effective against multiple coronaviruses.

To our knowledge, there are two recent reports describing cell-based assays that have been used to monitor 3CL^{Pro} from multiple human coronaviruses. We believe that our one vector system is distinct from these two systems with its simplicity and ability to be easily and quickly adapted. One assay described by others involves the use of two vectors, one has vesicular stomatitis virus (VSV) genome with the essential L protein replaced by a fluorescent protein reporter and a second vector encodes 3CL^{Pro} linked to the L protein [26]. When the 3CL^{Pro} cleaves and releases the L protein in cells coexpressing both vectors, VSV can replicate and spread to neighboring cells, which is monitored by the fluorescent spots in the culture. A modified system contains one vector expressing VSV with the P protein replaced by a reporter, and a second vector expressing 3CL^{Pro} embedded within a P protein. Inhibition of 3CL^{Pro} activity preserves the P protein function and allows VSV replication. In these systems, detection of a novel 3CL^{Pro} activity requires (1) generation of a new chimeric protein(s) that retain the 3CL^{Pro} and P protein functions, (2) coexpression of two vectors at appropriate levels to support VSV replication and (3) equipment to count fluorescent cell clusters such as a high-content imaging system [26].

The other reported system relies on the transactivation of a luciferase reporter cassette in HEK293T cells [24]. To detect 3CL^{Pro} activity, two plasmids expressing 3CL^{Pro} and the transactivator protein, are transfected into the luciferase gene-containing HEK293T cell line. 3CL^{Pro} cleavage of the fusion protein activates the transactivator, which then turns on the promoter to express luciferase. Thus, in this system, a cell line expressing an inducible reporter is needed, and to examine 3CL^{Pro} activity in other cell types requires the generation of a new cell line [24].

In conclusion, we demonstrate that the luciferase complementation reporter assay can be easily adapted to screen inhibitors for 3CL proteases of all seven currently known coronaviruses that infect humans. Moreover, this system could be modified to assess the activity/function and screen inhibitors of proteases from different coronavirus species, as well as other viruses that utilize a viral protease for their replication, such as enteroviruses, flaviviruses, adenoviruses, etc. The flexibility displayed by our assay to easily replace not only the protease but also substrate in order to quickly optimize signal readout offers a robust starting point for screening pan-coronavirus inhibitors as well as inhibitors of novel emerging coronaviruses with pandemic potential.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/v18020234/s1>, Figure S1: Cell viability of 293T cells after SAS vector transfection and GC376 treatment; Figure S2: Western blot and quantification of target site cleavage by HCoV 3CL proteases; Figure S3: Dose–response analysis of GC376 inhibition of 3CL^{Pro} activity measured by NanoLuc reporter assay.

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