Dear Editor,

The Coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The genome of this virus encodes two overlapping polyproteins, pp1a and pp1ab (Wu et al., 2020; Zhou et al., 2020). The functional polypeptides are released from those two polyproteins by extensive proteolytic events, mostly mediated by main protease (Mpro, also known as 3CLpro). It cleaves the polyproteins at no less than 11 conserved sites to produce non-structure proteins (Nsp) (Wu et al., 2020), and no human protease has been found to share similar cleavage specificity (Wu et al., 2020). Since these cleavage events play an essential role in viral replication and pathogenesis, Mpro is considered as one of the most attractive targets for drugs development to combat the ongoing COVID-19 pandemic (Pillayar et al., 2016).

Gaussia Luciferase (GLuc) is a naturally secreted luciferase (Luc) from the deep sea copepod Gaussia princeps (Bowlby and Case, 1991). It is an ideal generic reporter because it offers greatly increased bioluminescence than other commonly used Lucs (Bartok et al., 2013). It was reported that the fusion of mouse pro-interleukin (IL)-1β on the N-terminal of GLuc can inhibit its catalytic activity because pro-IL-1β can lead protein aggregates (Bartok et al., 2013).

To date, no Mpro inhibitor has been approved for SARS-CoV-2. In this study, we present a transgene-encoded biosensor that monitors thecleavage activity of SARS-CoV-2 Mpro in living cells. As shown in Fig. 1A, a fusion protein constructed of mouse pro-IL-1β and GLuc lacking secretion signal (i-GLuc) was generated as the negative control. A canonical Mpro cleavage site AVLQ15GFGR was inserted into i-GLuc at different positions to generate three biosensors to monitor Mpro activity. Theoretically, pro-IL-1β renders GLuc enzyme inactive in these biosensors, but Mpro can cleave the biosensors, leading to protein monomerization and GLuc activation.

Western blot assay was performed in HEK293T cell to test the cleavage of biosensors by Mpro (Supplementary Material). As expected, when co-expressed with SARS-CoV-2 Mpro, no cleaved band of i-GLuc appeared, but cleaved Flag-tagged bands of three biosensors were observed (Fig. 1B), indicating Mpro processed the cleavage site within the biosensors effectively and specifically.

Luciferase assay was performed to validate the efficiency of biosensors. As shown in Fig. 1C, i-GLuc generated similar bioluminescence in HEK293T cell with or without Mpro. In contrast, when Mpro was co-expressed with biosensors, an obvious increase of the GLuc signal occurred. The signal-to-background ratios (SBRs) of three biosensors were 3.97, 13.10, and 11.60, indicating Mpro-mediated cleavage activated GLuc activity in biosensors. Since the i-MS-GLuc2 construct presented the highest SBR, it was used for the succeeding experiments. The Linear range of i-MS-GLuc2was presented in Supplementary Material. GC376 and walrycin B obviously decreased the bioluminescence of the Mpro-active biosensor (Fig. 1D). Further, we tested the effects of these two drugs on the negative control iGLuc. GC376, anacardic acid, GW5074, and walrycin B (Jin et al., 2020; Zhu et al., 2020), were analyzed by the biosensor co-expressed with SARS-CoV-2 Mpro. GC376 and walrycin B obviously decreased the bioluminescence of the Mpro-active biosensor (Fig. 1D). Further, we tested the effects of these two drugs on the negative control iGLuc. GC376 had no effect on the signal of iGLuc, but the iGLuc signal decreased as the concentration of walrycin B increased (Fig. 1E and F). It may be caused by the cytotoxicity of walrycin B. Cell Counting Kit-8 (CCK-8) assay was performed on HEK293T cells to confirm this hypothesis (Supplementary Figure S2). GC376 showed no cytotoxicity on HEK293T cells even at a

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high concentration of 20 μmol/L, while walrycin B showed obvious cytotoxicity at the concentration of 0.1 μmol/L.

To facilitate anti-viral drugs screening, two reporter cell lines Vero E6-i-MS-GLuc2 and Vero-i-MS-GLuc2 that stably express i-MS-GLuc2 biosensor and sensitive to SARS-CoV-2 were constructed. Overexpressed Mpro leaded to GLuc signal increased in both reporter cell lines as predicted (Fig. 1G and Supplementary Figure S3). To test the sensitivity of the reporter cell line against live virus, Vero E6-i-MS-GLuc2 was infected with SARS-CoV-2 at MOI of 0.0005–0.1. Fig. 1H shows a good correlation between viral titer and GLuc signal. Cells infected with virus at an MOI of 0.1 showed significant GLuc signal increase and evident cytopathic effect (CPE), but no cell detachment. Thus, we used this MOI in the drug inhibition experiments.

GC376 was used to validate the cell line-based antiviral drugs screening system. We detected the inhibitory effects of GC376 at different concentrations on SARS-CoV-2 in reporter cell line via luciferase assay. As expected, the GLuc signal decreased as drug concentration increased from 0 μmol/L to 20 μmol/L (Fig. 1I), indicating the inhibition of virus by this drug. Thus, this reporter cell line could be applied as an antiviral drugs screening tool.

In this study, we presented a transgene-encoded GLuc biosensor and a cell-based antiviral drugs screening system that allowed the monitoring of SARS-CoV-2 Mpro activity in living cells instead of in vitro solutions. Except for biosensor based on GLuc, some other fluorescent reporters to detect SARS-CoV-2 Mpro activity were developed (Froggatt et al., 2020; Rawson et al., 2021). All of these systems are suitable for high-throughput assay. EGFP is visible and luciferase is more sensitive and quantifiable (Supplementary Table S1). Compared with other enzymatic activity assays, GLuc assay is highly sensitive, quantifiable, and easy to operate, suitable for high-throughput screening. However, the effect of drugs on negative control iGLuc should be tested, and internal control should be involved to avoid uncertain results caused by

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**Fig. 1.** Development of a biosensor assessing SARS-CoV-2 main protease activity in living cells for antiviral drugs screening. A Construction of i-GLuc and three biosensors. B Cleavage efficacy of SARS-CoV-2 Mpro on i-GLuc and biosensors detected by Western blot (WB). C Cleavage efficacy of SARS-CoV-2 Mpro on i-GLuc and biosensors detected by luciferase assay. HEK293T cells were transfected with pHA-Mpro and p-i-GLuc or biosensors expression plasmids. Cells were lysed and subjected to WB at 24 hours post-transfection. D The inhibition of five potential inhibitors against Mpro detected by the biosensor. HEK293T cells were transfected with pHA-Mpro and p-i-MS-GLuc2-Flag. At 4 hours post-transfection, cells were treated with DMSO, anacardic acid, disulfiram, GC376, GW5074 and walrycin B respectively, drug concentration = 20 μmol/L. E-F The effect of walrycin B and GC376 on negative control iGLuc. HEK293T cells were transfected with pHA-Mpro and p-i-GLuc-Flag. At 4 hours post-transfection, cells were treated with walrycin B or GC376 at different concentration. G The cleavage effect of SARS-CoV-2 Mpro in VERO-E6-i-MS-GLuc2 cells detected by luciferase assays. Cells were transfected with empty vector (EV) or pHA-Mpro. At 24 hours post-transfection, cells were lysed and subjected to luciferase assay. H The correlation between the SARS-CoV-2 viral titer and luciferase activity in VERO-E6-i-MS-GLuc2 cells. Cells were infected with SARS-CoV-2 at an MOI of 0.1. At one hour post-infection, cells were treated with GC376 at 0, 2, and 20 μmol/L. Luciferase activity was tested at 24 hours post-infection. These results are presented as the means ± SD of triplicate measurements. Error bars show the standard deviation of three experiments. Student’s t-test; ns, non-significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
some interference factors, such as drug cytotoxicity. In addition, our system has the potential to be applied in animal models because GLuc can be used in small animals as a marker. Therefore, it should be extremely useful for studying viral protease and screening novel anti-viral agents.

Footnotes

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