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Label-free duplex SAMDI-MS screen reveals novel SARS-CoV-2 3CLpro inhibitors

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ABSTRACT

The 3-chymotrypsin-like cysteine protease (3CLpro) of severe acute respiratory syndrome conoravirus 2 (SARS-CoV-2) remains a promising therapeutic target to combat COVID-19. Our group recently described a novel duplexed biochemical assay that combines self-assembled monolayers of alkanethiolates on gold with matrix assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry (MS) to simultaneously measure 3CLpro and human rhinovirus 3C protease activities. This study describes applying the assay for the completion of a high-throughput duplexed screen of 300,000 diverse, drug-like small molecules in 3 days. The hits were confirmed and evaluated in dose response analyses against recombinant 3CLpro, HRV3C, and the human Cathepsin L proteases. The 3CLpro specific inhibitors were further assessed for activity in cellular cytotoxicity and anti-viral assays. Structure activity relationship studies informed on structural features required for activity and selectivity to 3CLpro over HRV3C. These results will guide the optimization of 3CLpro selective inhibitors to combat COVID-19 along with antiviral compounds against coronaviruses and rhinoviruses.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for the coronavirus disease 2019 (COVID-19) pandemic. SARS-CoV-2 is an enveloped, positive sense single-stranded RNA (ssRNA) virus that belongs to the B-coronavirus genus of the Coronaviridae family, which also includes MERS and SARS. Most infected individuals experience mild symptoms similar to the common cold or remain asymptomatic, while ~20% develop severe to critical manifestations defined by respiratory failure, septic shock, and/or multiple organ dysfunction (Wiersinga and Prescott, 2020). By early 2021, COVID-19 was the leading cause of death in the United States (U.S.) (https://www.healthsystemtracker.org/brief/covid-19-is-the-number-one-cause-of-death-in-the-u-s-in-early-2021/) and fourth worldwide (http://deathmeters.info). The rapid development and deployment of effective vaccines (Wibawa, 2021) is a viable frontline defense against COVID-19. However, some individuals may not have access to vaccines or be willing to take them. In addition, the evolution of SARS-CoV-2 and presentation of distinct variants has presented a challenge for vaccine effectiveness, supporting the need for alternative therapeutic strategies.

The first antiviral drug authorized under Emergency Use Authorization (EUA) by the United States FDA for the treatment of COVID-19 patients was Remdesivir (Ison et al., 2020). The efficacy of Remdesivir reduced recovery from 15 days to 11 days, but had no significant effect on mortality (Beigel et al., 2020). Remdesivir requires hospitalization and intravenous injection, limiting access to growing patient numbers. More recently, Paxlovid and Molnupiravir have been granted EUA by the US FDA. Similar to Remdesivir, Molnupiravir targets the viral RNA polymerase (Kabinger et al., 2021). Conversely, Paxlovid targets the SARS-CoV-2 3C-like cysteine protease (3CLpro), an essential enzyme for viral replication (Boras et al., 2021). Without a human-homolog counterpart, 3CLpro offers an attractive therapeutic target to combat COVID-19, motivating further drug discovery efforts to address the increased need for therapeutic intervention. To date, drug discovery strategies for 3CLpro have largely focused on structure-based design and virtual screening efforts or limited high-throughput screening (HTS) of small molecules, including opportunities for repurposing approved drugs (Ghosh et al., 2007; Jin et al., 2020; Lee et al., 2014; Mody et al., 2021). Early inhibitors mimicked 3CLpro substrate specificity (Ghosh et al., 2005; Jain et al., 2004; Xue et al., 2008; Yang et al., 2006; Zhang et al., 2007). These peptidomimetics often featured a motif that forms a covalent bond with the catalytic Cys145, while others have been reported to function as a reversible covalent mechanism (Fu et al., 2020; Ma et al., 2020). Small molecule inhibitors have been reported as alternatives to peptidomimetics, with early examples largely operating through covalent mechanisms (Jacobs et al., 2013; Turlington et al.,

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2013), and a recent report highlighted a natural product non-covalent inhibitor of 3CLpro, offering yet another therapeutic modality for exploration (Su et al., 2020). Along with these important studies, identifying novel chemical matter with pharmacophore-like properties that operate through non-covalent mechanisms of action to minimize unwanted immunogenicity and off-target effects remains critical.

One of the challenges with protease drug discovery is a lack of suitable high-throughput assay formats. Traditional biochemical assays have relied on FRET readouts (Dai et al., 2020; Jin et al., 2020), which are prone to false-positive and false-negative hits due to optical interference with library compounds (Gurard-Levin et al., 2020; Kaeberlein et al., 2005). Our group has recently reported a label-free, high-throughput assay for measuring 3CLpro activity (Gurard-Levin et al., 2020). The approach, termed SAMDI-MS, combines self-assembled monolayers (SAMs) and MALDI-TOF mass spectrometry (MS) to offer a powerful approach for measuring biochemical and binding activities (Buker et al., 2020; Gurard-Levin and Mrksich, 2008; Gurard-Levin et al., 2011; Liu et al., 2021; Mrksich, 2008; Scholle and Gurard-Levin, 2021; Scholle et al., 2021a, 2021b; VanderPorten et al., 2017). An added benefit of the SAMDI-MS assay is the multiplexing capability, exemplified by the recent report of a duplexed SAMDI-MS assay to evaluate the potency and selectivity of reported 3CLpro inhibitors against two distinct proteases simultaneously (Liu et al., 2021). In this study, we apply the label-free duplexed SAMDI-MS assay to screen 300,000 small molecules from a diverse, high purity, pharmacophore-like library to identify novel and selective 3CLpro inhibitors. The screen was completed in a duplexed format to inform not only on 3CLpro activity, but selectivity over the human rhinovirus 3C protease. The identified 3CLpro hits were confirmed in follow-up validation and dose response analyses in a SAMDI-MS and FRET format, along with cross reactivity assays against the human rhinovirus 3C protease (HRV3C) and human protease Cathepsin L. Critical structural features were analyzed to inform on structure activity relationships. Lead compounds were further tested in cellular cytotoxicity and antiviral assays resulting in on target antiviral activity. By sharing the results with the scientific community, including the structures of 3CLpro inhibitors, this study aims to encourage further evaluation and optimization of 3CLpro inhibitors to strengthen the fight against COVID-19 and future pandemics.

The schematic for the high-throughput duplexed SAMDI-MS assay for SARS-CoV-2 3CLpro and HRV3C protease is presented in Fig. 1A. Protease reactions were initiated by adding the 3CLpro peptide substrate (Ac-TSAVLQSGFRKK(biotin)-NH_2) and the HRV3C substrate (Biotin-REEVLFQGPG-NH_2) to a low volume 384-well plate with 3 nM 3CLpro (final) and 6 nM HRV3C (final). The peptide sequences were selected based on historical data for substrate specificity (Fan et al., 2005; Gurard-Levin et al., 2020). Importantly, using kinetically balanced conditions, where substrate concentrations equal the K_M values and enzyme activity is linear, the two enzymes exhibit specific activity for their cognate substrate (Liu et al., 2021). After quenching activity using formic acid (0.5% final) and adding internal standards for quantitation (Liu et al., 2021), automated liquid handlers transfer the reactions to Neutravidin-presenting self-assembled monolayer biochip arrays. The Neutravidin serves to specifically immobilize the biotinylated substrates, products, and internal standards, while the tri(ethylene glycol) terminated alkanethiolates minimize non-specific protein adsorption (Mrksich and Whitesides, 1996). The SAMDI-MS spectra show peaks at

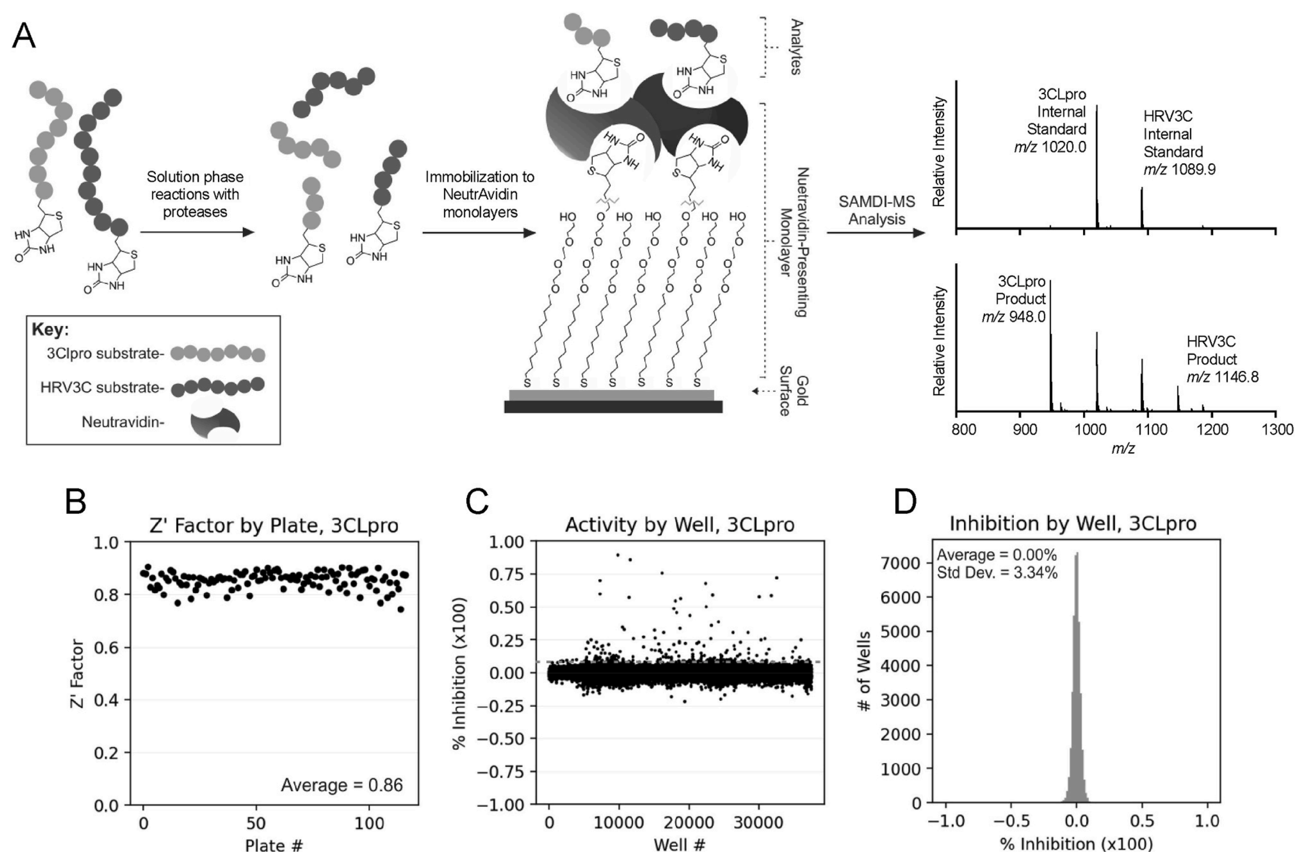

Fig. 1. Schematic of the duplexed SAMDI-MS HTS. (A) *left* 3CLpro and HRV3C enzymes incubate with their selective substrates in a homogenous solution reaction in the presence of compounds. The reactions are quenched and internal standards added prior to transfer to Neutravidin presenting monolayers in a high-density biochip array format. *Right* In the absence of enzyme, SAMDI-MS analysis reveals peaks at *m/z* 1020.0 and *m/z* 1089.9 corresponding to the internal standards for 3CLpro and HRV3C, respectively. In the presence of enzyme, additional peaks are observed at *m/z* 948.0 and *m/z* 1146.8 corresponding to the products of 3CLpro and HRV3C activity, respectively. (B) Z-factors of the 3CLpro activity across the screen of 300,000 compounds. (C) The % inhibition of each compound against 3CLpro activity across the screen. (D) Histogram showing the distribution of 3CLpro inhibitors across the screen.

m/z 948.0 and *m/z* 1146.8, corresponding to the enzyme products of 3CLpro and HRV3C respectively, and peaks at *m/z* 1020.0 and *m/z* 1089.9 represent the respective 3CLpro and HRV3C internal standards.

The primary duplexed SAMDI-MS screen of 300,000 small molecules was complete in 3 days and represents, to the best of our knowledge, the largest reported biochemical HTS for 3CLpro and HRV3C. The 300,000 compounds derive from a diverse, pharmacophore-like library synthesized in 2020 with an average purity of >95%. The library was pooled with eight compounds per well prior to screening to further accelerate hit finding efforts, where each compound was screened at a final concentration of 12.5 μM (1% DMSO final). Each plate included 32 control wells for 0% inhibition (DMSO vehicle) and 32 wells for 100% inhibition (pre-quenched with 0.5% final formic acid). The controls allow an assessment of robustness of the assay, which was consistent across each plate throughout the screen with an average Z-factor for 3CLpro of 0.86 (Fig. 1B) and an average robust Z-factor of 0.70 for HRV3C (Supplementary Figure S1A). Hit calling was determined using a threshold of 3 standard deviations above the average inhibition across the screen (0.0% and 7.9% for 3CLpro and HRV3C, respectively) (Fig. 1C and D, Supplementary Figure S1B) resulting in 140 pools for 3CLpro and 35 pools for HRV3C achieving the hit threshold criteria.

To identify the specific hits, the eight compounds from each well were analyzed as individual compounds at 12.5 μM in duplicate in the duplexed SAMDI-MS assay. The quality of the assay is exemplified by the reproducibility of the replicate data (slope = 0.976 for 3CLpro) (Fig. 2A and B). Validated hits were determined as compounds that achieved the respective inhibition thresholds defined in the primary screen. In line with the ratio of the initial 3CLpro and HRV3C hits, 93 compounds from the 140 pooled wells inhibited 3CLpro (67% validation rate based on well) and 30 inhibited HRV3C (85% validation rate), and only 7 compounds exhibited activity on both proteases (Fig. 2C), consistent with the distinct catalytic sites of these two enzymes (Liu et al., 2021). The high validation rate exemplifies the benefits of label-free assays including the elimination of false positives and negatives from optical interference of library compounds and supports the robustness and reproducibility of the SAMDI-MS approach. Moreover, by generating a signal for the substrate and product, the SAMDI-MS assay also avoids calling false positives for compounds that may interfere with immobilization, for example by biotin mimetics, as this would impact both substrate and product equally. The validated hits were next analyzed in a 10-point dose response format in duplicate in both the duplexed SAMDI-MS assay and in a human Cathepsin-L assay using a traditional FRET approach using previously described conditions (Liu et al., 2021) (Supplementary Figure S2). The data highlights 24 3CLpro compounds and 19 HRV3C compounds that feature IC_{50} values < 25 μM and a Hill Slope within the range of 0.8 and 1.8 for their respective targets.

The 8 most potent 3CLpro and 4 most potent HRV3C inhibitors (compounds **1–11**, where compound **2** is similarly active on both enzymes) (Fig. 3) were reevaluated from fresh powder and the purity of each compound was determined by LC-MS (Table 1) prior to dose response analysis in the duplexed SAMDI-MS assay human Cathepsin L FRET assay (Supplementary Figure S3), with IC_{50} values highlighted in Table 1. To validate hits in a secondary assay, a selection of compounds was analyzed for 3CLpro inhibition in a traditional FRET based assay (Supplementary Figure S4, Supplementary Table S3). While the majority of compounds exhibit similar behavior and IC_{50} values in the SAMDI-MS and FRET formats, compounds **7**, **8**, and **30** generate high fluorescent signals, particularly at concentrations >10 μM. These data represent would-be false negatives if the screen were run in the FRET format as the high fluorescent signal from the compound itself masks the potential loss of fluorescent signal due to inhibition of the enzyme. Conversely, compound **10** exhibits an IC_{50} curve in the FRET format, but not the SAMDI-MS assay. These data are indicative of a false positive due to compound quenching of the fluorescent signal, as previously observed with the reported 3CLpro inhibitor Shikonin (Gurard-Levin et al., 2020). Taken together, these data showcase the importance of label-free assays to generate quality data to drive drug discovery.

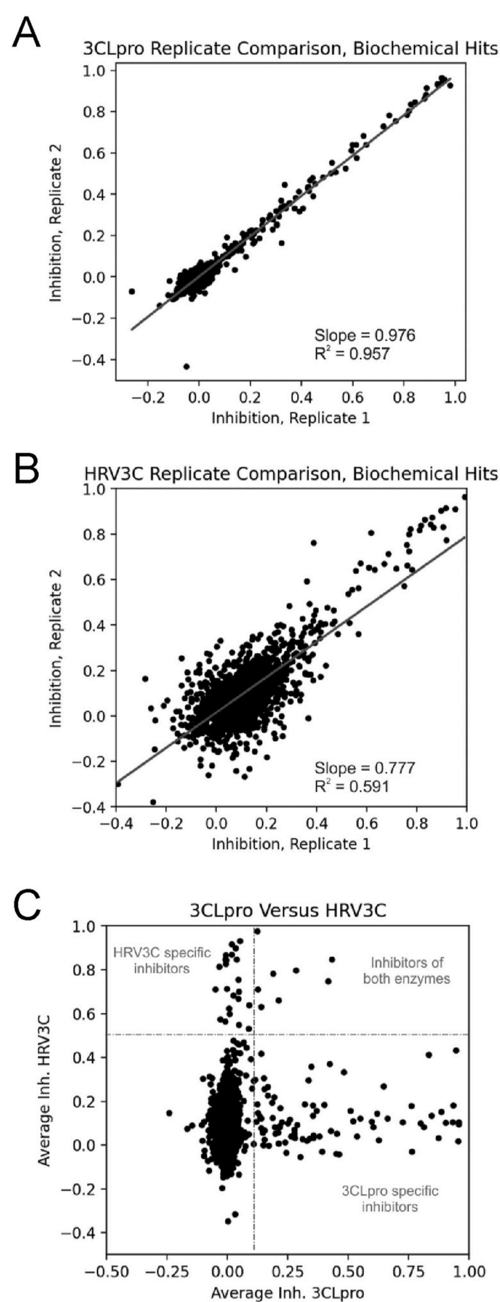

Fig. 2. Hit validation using duplexed SAMDI-MS assay. Replicate data evaluating hits from the primary screen for (A) 3CLpro and (B) HRV3C in the duplex format. (C) Plot of primary hits to demonstrate 3CLpro and HRV3C selectivity. Inhibition values determined by multiplying axis labels by 100.

The 11 compounds were then tested in a 5-point dose response analysis against a SARS-CoV-2/VeroE6-EGFP cell line to measure cytotoxicity (standard MTS assay) (Jochmans et al., 2012) and anti-viral activity as previously described (Gurard-Levin et al., 2020; Ivens et al., 2005), including Remdesivir as a control. In line with previous reports, Remdesivir exhibited an EC_{50} of 1.84 μM in the anti-viral assay (Fig. 4A). While the HRV3C selective inhibitors **9–11** did not generate an anti-SARS-CoV-2 response as anticipated (Table 2), the 3CLpro selective inhibitor **4** exhibited an EC_{50} of 14.9 μM with no observed cytotoxicity (Fig. 4B). Structurally similar compounds **1** and **3** were not active in cells (Table 2), although whether the compounds are cell permeable remains an open question.

The anti-viral activity of compound **4** motivated a study of structure

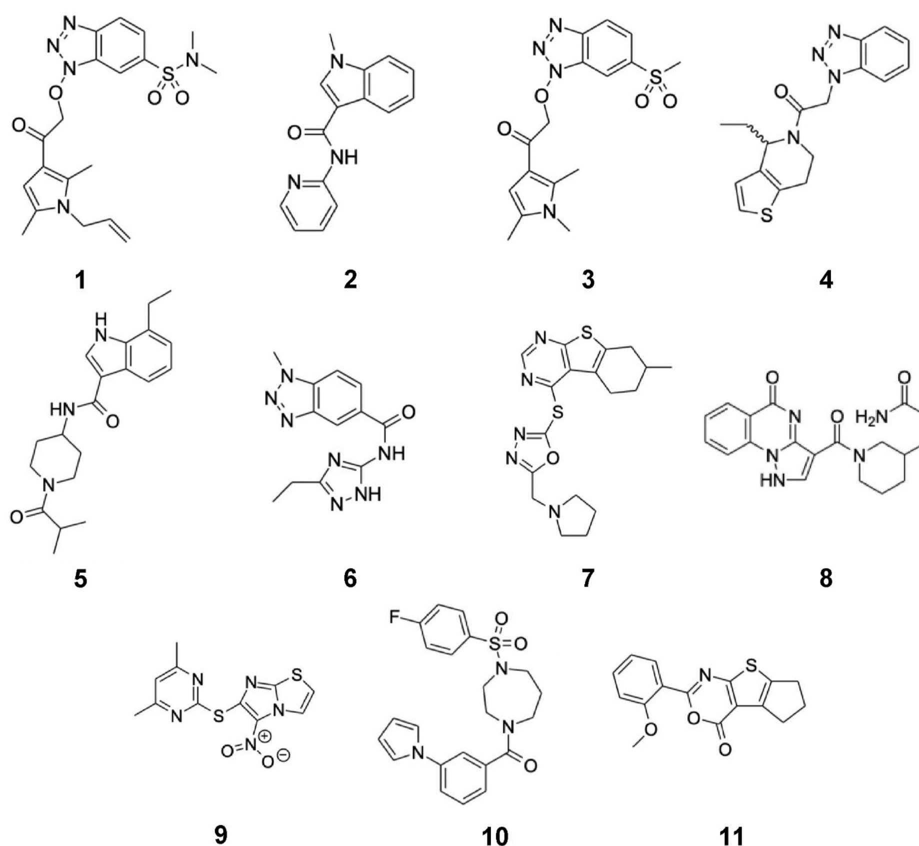

Fig. 3. Structures of identified 3CLpro and HRV3C inhibitors.

Table 1
Summary of inhibition of 3CLpro and HRV3C in duplexed SAMDI-MS assay and Cathepsin L in FRET assay. ND = not determined.

Cmpd #	% Purity	3CLpro IC_{50} (μM)	3CLpro Hill Slope	HRV3C IC_{50} (μM)	HRV3C Hill Slope	CathL IC_{50} (μM)	CathL Hill Slope
1	96	1.67	1.65	>100	ND	>100	ND
2	91	4.53	1.28	2.32	1.44	>100	ND
3	97	4.67	1.91	>100	ND	>100	ND
4	100	5.48	1.54	>100	ND	>100	ND
5	95	6.45	1.77	28.49	0.96	>100	ND
6	97	7.37	1.48	57.03	1.34	>100	ND
7	95	14.76	1.27	>100	ND	>100	ND
8	95	21.2	1.31	>100	ND	>100	ND
9	100	82.2	1.4	4.94	1.31	55.4	1.33
10	100	>100	ND	1.67	1.45	66.9	1.19
11	93	>100	ND	3.94	1.08	>100	ND

activity relationships to inform on critical scaffolds. There are two main structural motifs: a 1, 2, 3, benzotriazole and a bicyclic moiety integrating piperidine and thiophene rings. To assess the importance of the piperidine thiophene rings, 12 compounds that maintained the benzotriazole were tested with varying substituents (Supplementary Table S1). Each compound was obtained from fresh powder and the purity verified by LC-MS (data not shown) prior to testing. The data shows that replacing piperidine and thiophene rings with planar aromatic groups leads to diminished activity, while the pyrrole group on compound **12** exhibited an IC_{50} of 1.32 μM (0.35 μM by FRET (Supplementary Figure S4)), a similar potency to compounds **4**, **3**, and **1**, where the latter two feature the pyrrole group. Next, substituents to the piperidine and thiophene scaffold were explored over 6 compounds and none of them inhibited 3CLpro (Supplementary Table S2). These data are in line with previous reports of similar benzotriazole compounds (Wu et al., 2006) and consistent with the weaker potency of 3CLpro inhibitors **7–9** that lack the 1, 2, 3, benzotriazole scaffold. Taken together, the structure activity relationships point to the piperidine and thiophene bicyclic scaffold and the 1, 2, 3 benzotriazole scaffold as important functional groups that contribute to 3CLpro inhibition.

Compound **4** features a chiral center with the ethyl group on the piperidine ring. Following enantiomer purification (data not shown), the enantiomers **4-R** and **4-S** were tested in the duplexed SAMDI-MS and FRET assays. The SAMDI-MS and FRET data highlight that the **4-S** enantiomer is at least 25-fold more potent than the **4-R** enantiomer (Fig. 4C, Supplementary Figure S4), suggesting that this substituent may occupy a certain binding pocket of the enzyme. These data offer a launching point for further optimization to increase potency through medicinal chemistry efforts. Taken together, the high-throughput screen using the duplexed SAMDI-MS assay rapidly identified a novel 3CLpro inhibitor with selectivity over the rhinovirus 3C protease and human cathepsin L and with anti-viral activity in cells. Compound **4-S**, with pharmacophore-like properties is structurally distinct from peptidomimetic clinical candidates, which may provide a new avenue for SARS-CoV-2 therapeutics, prophylactics, and antiviral compounds against novel coronaviruses to help neutralize future pandemics.

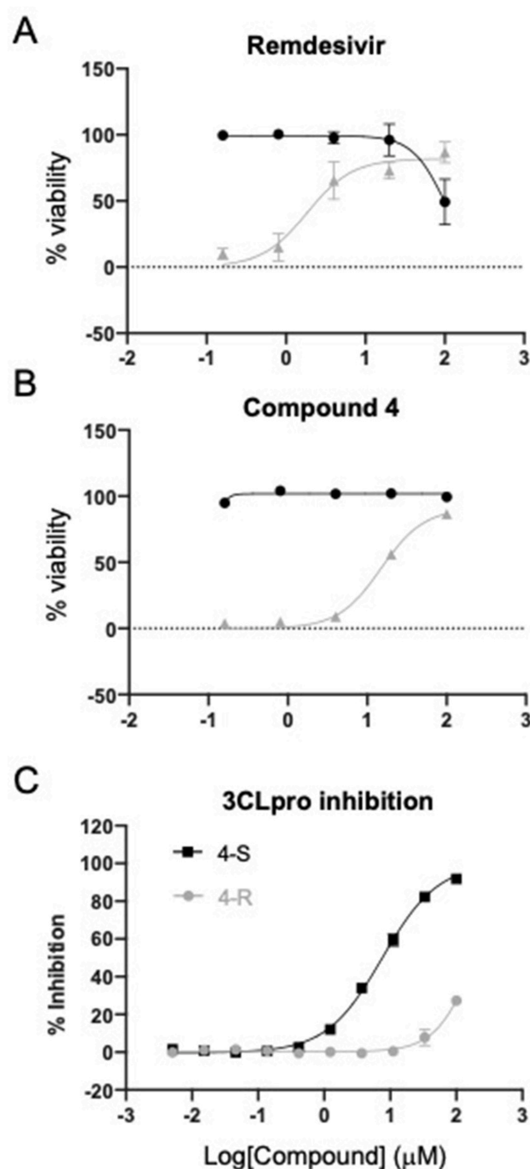

Fig. 4. Evaluation of Remdesivir control (A) and Compound 4 (B) to measure CC_{50} (cytotoxicity) (black circles) and EC_{50} (anti-viral activity) (grey triangles) in cellular assays. (C) IC_{50} measurements of compound 4-S (black squares) and compound 4-R (grey circles) by SAMDI-MS. Experiments were performed in duplicate and error bars represent standard deviation.

Table 2
Summary of cytotoxic and antiviral effect against human SARS-CoV-2 in Vero-GFP cells in culture. Values represent the average of duplicate data.

Cmpd #	CC_{50} (μM)	EC_{50} (μM)
Remdesivir	**~100**	**1.84**
1	**>100**	**>100**
2	**50.1**	**>100**
3	**>100**	**>100**
4	**>100**	**14.9**
5	**>100**	**>100**
6	**>100**	**>100**
7	**14.9**	**>100**
8	**>100**	**>100**
9	**17.1**	**>100**
10	**>100**	**>100**
11	**81.5**	**>100**

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.antiviral.2022.105279.

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