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Discovery of PL^{pro} and M^{pro} Inhibitors for SARS-CoV-2

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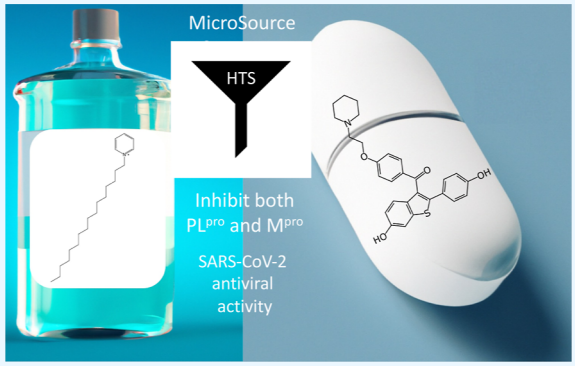
ABSTRACT: There are very few small-molecule antivirals for SARS-CoV-2 that are either currently approved (or emergency authorized) in the US or globally, including remdesivir, molnupiravir, and paxlovid. The increasing number of SARS-CoV-2 variants that have appeared since the outbreak began over three years ago raises the need for continual development of updated vaccines and orally available antivirals in order to fully protect or treat the population. The viral main protease (M^{pro}) and the papain-like protease (PL^{pro}) are key for viral replication; therefore, they represent valuable targets for antiviral therapy. We herein describe an in vitro screen performed using the 2560 compounds from the Microsource Spectrum library against M^{pro} and PL^{pro} in an attempt to identify additional small-molecule hits that could be repurposed for SARS-CoV-2. We subsequently identified 2 hits for M^{pro} and 8 hits for PL^{pro}. One of these hits was the quaternary ammonium compound cetylpyridinium chloride with dual activity (IC_{50} = 2.72 ± 0.09 μM for PL^{pro} and IC_{50} = 7.25 ± 0.15 μM for M^{pro}). A second inhibitor of PL^{pro} was the selective estrogen receptor modulator raloxifene (IC_{50} = 3.28 ± 0.29 μM for PL^{pro} and IC_{50} = 42.8 ± 6.7 μM for M^{pro}). We additionally tested several kinase inhibitors and identified olmutinib (IC_{50} = 0.54 ± 0.04 μM), bosutinib (IC_{50} = 4.23 ± 0.28 μM), crizotinib (IC_{50} = 3.81 ± 0.04 μM), and dacominitinib (IC_{50} = IC_{50} 3.33 ± 0.06 μM) as PL^{pro} inhibitors for the first time. In some cases, these molecules have also been tested by others for antiviral activity for this virus, or we have used Calu-3 cells infected with SARS-CoV-2. The results suggest that approved drugs can be identified with promising activity against these proteases, and in several cases we or others have validated their antiviral activity. The additional identification of known kinase inhibitors as molecules targeting PL^{pro} may provide new repurposing opportunities or starting points for chemical optimization.



INTRODUCTION

It has been over three years since the initial outbreak of SARS-CoV-2 in Wuhan, China, in November 2019 which caused the COVID-19 disease,[1,2] and at the time of writing this paper there have been over 760 million cases and 6.8 million deaths[3] caused by this enveloped, positive-sense, single-stranded RNA betacoronavirus. In the USA, currently, only remdesivir is FDA-approved as an antiviral for COVID-19, while molnupiravir and paxlovid have emergency use authorizations for this virus. The lower efficacy of molnupiravir and concerns that it may induce mutations in patient DNA[4] have somewhat dampened enthusiasm for it. The continued emergence of new SARS-CoV-2 variants points to the need for the development of additional vaccines and oral antivirals if we are to overcome this virus globally.

Part of the challenge in SARS-CoV-2 and antiviral drug discovery, in general, is developing molecules against targets that may be less susceptible to resistance than the spike protein. There has been a long history of the development of protease inhibitors for human immunodeficiency virus (HIV) and hepatitis C virus (HCV).[5−7] For SARS-CoV-2, there are two proteases: the main protease (M^{pro} also known as 3CL^{pro}) and the papain-like protease (PL^{pro}).[8] The catalytic activity of these enzymes is key for viral replication, making their inhibition a compelling strategy for antiviral therapy for SARS-CoV-2.[8] For example, M^{pro} is inhibited by many known cysteine protease inhibitors, primarily via covalent modification of the active site cysteine and represents an opportunity for repurposing.[9−12]

Paxlovid consists of a combination of nirmatrelvir (PF-07321332), which is an inhibitor of the main protease (M^{pro}), and ritonavir, which is commonly used to improve the half-life. Mutations have been observed in M^{pro}, yet these have proved susceptible to nirmatrelvir. This drug was developed from an earlier covalent active-site-directed inhibitor of the SARS M^{pro}

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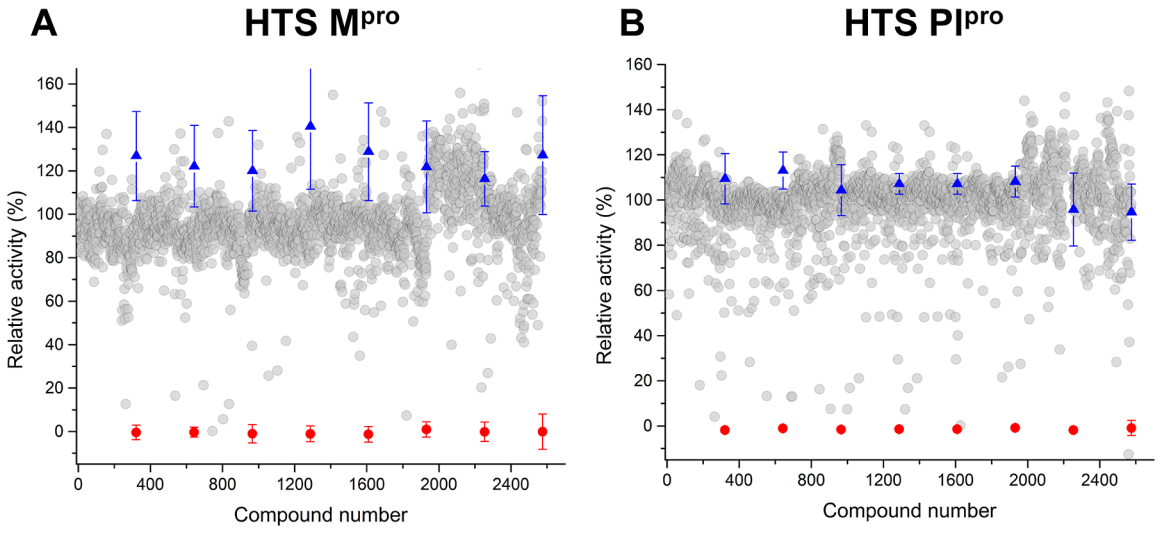

Figure 1. Scatter plots showing the data from the high-throughput screening (HTS) against M^{pro} (A) and PL^{pro} (B). Single-shot inhibition data are shown for compounds from the Microsource Spectrum collection containing 2560 compounds that were screened at 10 μM. Average $Z = 0.76 \pm 0.21$ for M^{pro} and average $Z = 0.67 \pm 0.13$ for PL^{pro}. Compounds' individual results are represented as gray spheres. Positive control averages are indicated as red spheres. Negative controls are indicated as blue triangles. Error bars are ± standard deviation from relative controls.

inhibitor PF-00835231, which contains an indole and was proposed as a P-glycoprotein (P-gp) substrate in Vero cells and hence had low activity and poor bioavailability.[13] It was recently determined that, in A549 + ACE2 cells, PF-00835231 is efficacious and suggests that P-gp may not be an issue after all.[14]

PL^{pro} cleaves peptide bonds to produce non-structural proteins. PL^{pro} also has an effect by countering the effect of viral infection on the immune response via deubiquitinating and deISGylating activities and releasing ubiquitin and ISG15, which suppresses the immune system.[15] Recent detailed reviews of drug discovery for SARS-CoV-2 PL^{pro} inhibitors have highlighted over 50 non-covalent and covalent molecules.[15] What is apparent from this body of research for PL^{pro} is the lack of an approved drug that displays both enzyme and antiviral activity. One potential issue with this target is the structural differences between the target in MERS-CoV and SARS-CoV that make a broad spectrum antiviral against this target difficult.[16] Target selectivity is also challenging because of other deubiquitinating enzymes and the need for counter screens against these cysteine proteases. Like any target, PL^{pro} is also susceptible to inhibitors that may be promiscuous, which might limit their value. While there have been some repurposing efforts for PL^{pro}, most of the molecules have been natural products or not evaluated in counter screens or orthogonal assays.[17] PL^{pro} therefore represents a relatively untapped target for treating COVID. We now describe the hits derived from our initial repurposing screens with PL^{pro} and M^{pro} and important observations relevant to ongoing studies with these molecules.

RESULTS

PL^{pro} and M^{pro}. We developed high-throughput assays for PL^{pro} and M^{pro} and used these to screen the Microsource Spectrum collection composed of 2560 compounds. The assays are suitable for screening (average $Z = 0.76 \pm 0.21$ for M^{pro} and average $Z = 0.67 \pm 0.13$ for PL^{pro}) when compounds were screened at 10 μM, and compounds able to inhibit at least 80% of enzyme activity were selected (Figure 1). From the initial screening, we identified eight compounds with activity against PL^{pro} and two compounds with activity against M^{pro} (Table 1). Surprisingly, we identified two compounds

Table 1. Compounds Identified against PL^{pro} and M^{pro} from the Microsource Spectrum Collection Library Screen[a]

compound	PL^{pro} IC_{50} (μM)	M^{pro} IC_{50} (μM)
raloxifene	3.28 ± 0.29	42.8 ± 6.7
cetylpyridinium chloride	2.72 ± 0.09	7.25 ± 0.15
cefonicid sodium	2.17 ± 1.2	
citicoline	1.35 ± 0.27	
colistin sulfate	2.94 ± 0.54	
β-lapachone	5.30 ± 0.29	
tanshinone iia sulfonate sodium	2.76 ± 0.28	
lobaric acid	10 ± 1.11	

[a]It should be noted that compounds such as β-lapachone and tanshinone may be potential false positives involved in redox cycling.[18]

that inhibited both proteases, and we followed them up by generating dose−response curves. Raloxifene inhibits PL^{pro} with an $IC_{50} = 3.28 \pm 0.29$ μM and M^{pro} $IC_{50} = 42.8 \pm 6.7$ μM, and cetylpyridinium chloride inhibits PL^{pro} with an $IC_{50} = 2.72 \pm 0.09$ μM and M^{pro} $IC_{50} = 7.25 \pm 0.15$ μM (Figure 2).

We previously identified vandetanib, a vascular endothelial growth factor (VGFR) inhibitor, with activity against SARS-CoV-2 in A549 − ACE2 cells, which blocked the cytokine storm in infected mice.[19] We then evaluated other kinase inhibitors against PL^{pro} and M^{pro} and showed that olmutinib inhibits PL^{pro} with an $IC_{50} = 0.54 \pm 0.04$ μM (Figure 3A), dacomitinib with an $IC_{50} = 3.33 \pm 0.06$ μM (Figure 3B), crizotinib with an $IC_{50} = 3.81 \pm 0.04$ μM (Figure 3C), and bosutinib with an $IC_{50} = 4.23 \pm 0.28$ μM (Figure 3D) were similarly active. None of the kinase inhibitors demonstrated inhibition with M^{pro}.

Cell Assays. Raloxifene[20−22] and cetylpyridium chloride[23,24] were previously described to have activity against SARS-CoV-2 in various cell types. We tested activity of 3 of the 4 kinase inhibitors due to limited resources such that

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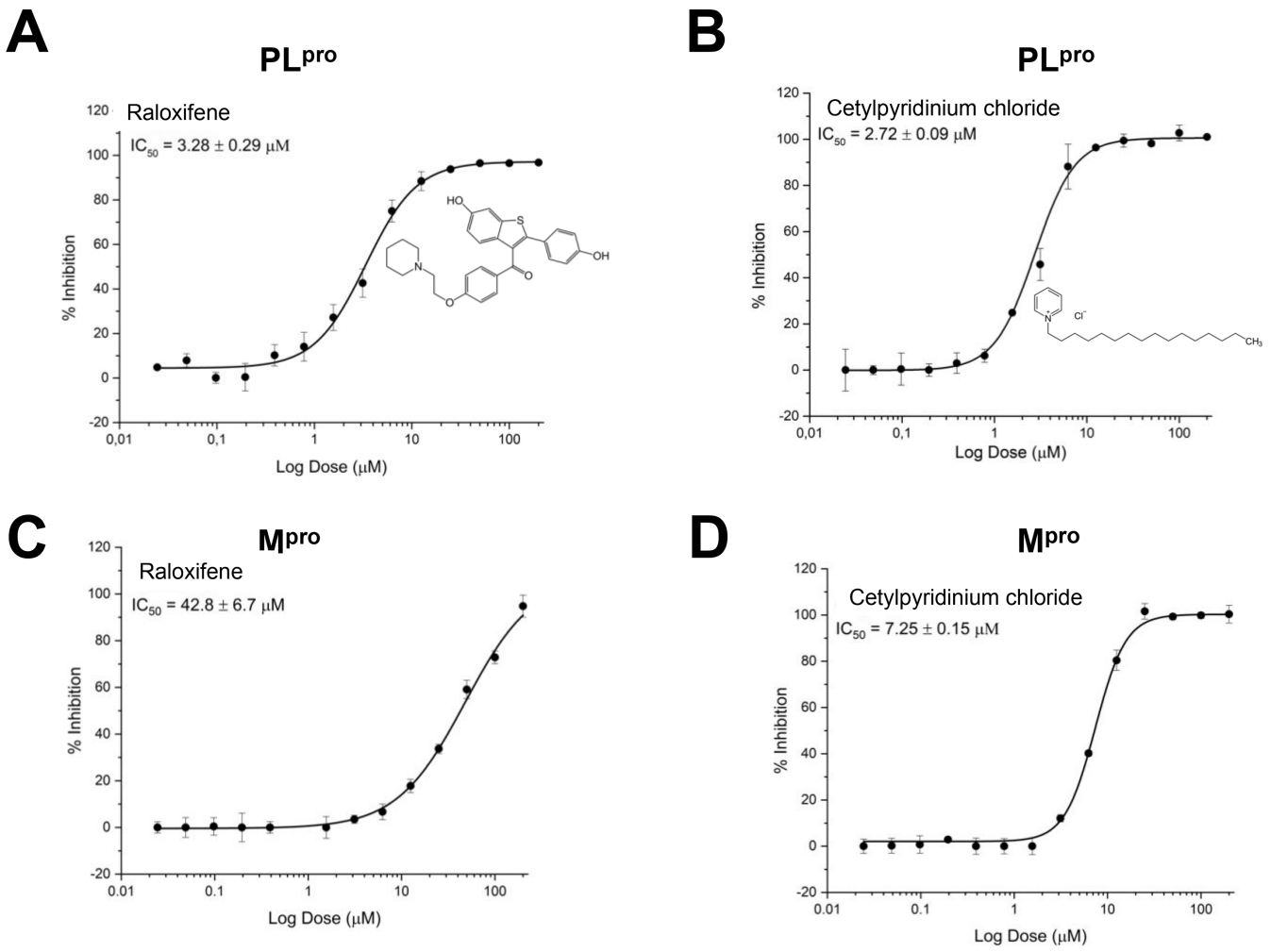

Figure 2. Dose−response curves for raloxifene and cetylpyridinium chloride tested against PL^{pro} (A,B) and M^{pro} (C,D). Both compounds were identified by screening the Microsource Spectrum collection. All experiments were performed in triplicate, and all data are expressed as the mean ± standard deviation.

bosutinib, crizotinib, and olmutinib were tested in Calu-3 cells available via the NIAID. Remdesivir was used as a general control for in vitro inhibition. Bosutinib showed an IC_{50} = 5.26 μM and CC_{50} = 6.06 μM, crizotinib showed an IC_{50} = 16.30 μM and CC_{50} = 5.09 μM, and olmutinib showed an IC_{50} = 9.76 μM and CC_{50} = 12.48 μM (Figure 4). All the kinase inhibitors tested showed substantial cell toxicity in Calu-3 cells.

DISCUSSION

Since the outbreak of SARS-CoV-2, there has been an enormous global effort to identify and develop potential antivirals using a vast number of techniques. Much of the early work included extensive use of computational approaches[25,26] as well as high-throughput screens, which has had admittedly mixed success, as briefly described here. Extensive effort has been applied toward developing M^{pro} inhibitors using a range of experimental approaches. For example, a ligand-based virtual screen of 790,000 compounds followed by structure-based screens for M^{pro} led to the testing of 30 compounds, of which 8 had high 10's μM IC_{50} and antiviral activity.[27] A docking and molecular dynamics approach was used to develop a covalent inhibitor of M^{pro} from a fragment hit, although the antiviral activity or selectivity was not assessed.[28] An in silico approach to screening over a billion compounds led to over 400 being synthesized, of which only 5 were active and ultimately resulted in a non-covalent inhibitor with a M^{pro} IC_{50} = 1 μM. Virtual pharmacophore and docking-based screening of over 8000 drugs against M^{pro} identified the kinase inhibitor nilotinib as a low μM antiviral in Vero cells but did not test inhibition of M^{pro} or PL^{pro}.[10] An ensemble docking approach was used with a M^{pro} to screen around 2000 natural products and identify 5 with high μM K_i values. Antiviral activity was not determined for these hits either.[29] A virtual repurposing screen of 8700 compounds against M^{pro} led to the identification of the preclinical molecule MG-132 with sub μM antiviral activity in Vero cells.[11] Docking FDA drugs in the M^{pro} structure identified dipyridamole (IC_{50} = 0.53 μM), which was active in Vero cells (EC_{50} ~ 0.1 μM).[30] An early screen of M^{pro} used 10,000 compounds, finding 7 hits including ebselen (IC_{50} = 0.67 μM) that was active in Vero cells (EC_{50} = 4.67 μM).[31] A screen of protease inhibitors against M^{pro} identified boceprevir (IC_{50} = 4.13 μM), which was similarly active in Vero cells (EC_{50} = 1.31 μM).[32] A high-throughput repurposing screen of over 6000 drugs against M^{pro} identified 50 hits and 8 with IC_{50} < 50 μM, and this data did not correlate with docking in the protein. Several of the best hits were hepatitis C inhibitors including boceprevir, but the antiviral activity was not determined.[9] The COVID moonshot initiative, which is a

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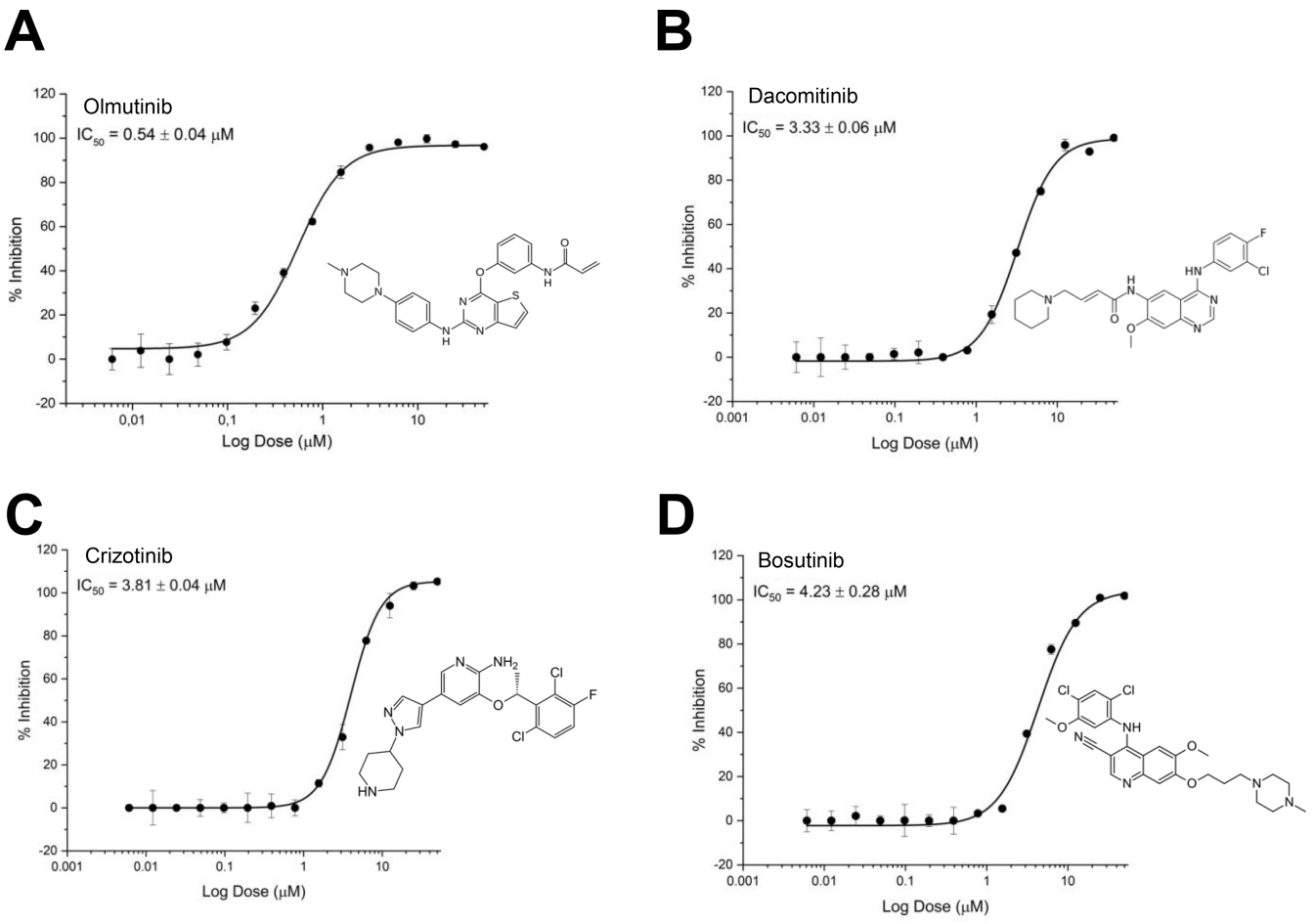

Figure 3. Dose−response curves of kinase inhibitors tested against PL^{pro}. (A) Olmutinib, IC_{50} 0.54 ± 0.04 μM, (B) Dacomitinib, IC_{50} 3.30 ± 0.06 μM, (C) Crizotinib 3.80 ± 0.04 μM, and (D) Bosutinib IC_{50} 4.20 ± 0.28 μM. All experiments were performed in triplicate, and all data are expressed as the mean ± standard deviation.

consortium that uses open science and open data to rapidly develop patent-free antivirals, reported the discovery of novel chemical scaffolds for M^{pro} active in biochemical and live virus assays, which were synthesized with model-generated routes.[33] Generative approaches have also been proposed for use with M^{pro} but to date this appears to have not been acted upon.[34]

These represent a small snapshot of the massive number of virtual or high-throughput screens undertaken to date. Others have used boceprevir for crystallography and structure-based design to identify further analogues with antiviral activity and in vivo efficacy in a mouse model of infection.[35] The flavonoid natural product baicalein is a sub μM inhibitor of M^{pro} and has been crystallized with this target from SARS-CoV-2.[36] Following crystallography of covalent inhibitors, low nM antiviral inhibitors selective (sub μM) for M^{pro} have been developed that reduced viral replication and viral load, increasing survival in SARS-CoV-2-infected mice.[37] The protease inhibitor 13b is less potent versus SARS-CoV-2 M^{pro} (IC_{50} = 0.67 μM) but shows activity in Calu-3 cells (EC_{50} = 4−5 μM).[38] Additional indole and indoline compounds were developed as M^{pro} inhibitors with antiviral activity against SARS-CoV-2.[39] There has also been extensive development of peptidic M^{pro} nM inhibitors such as tripeptides,[40] tripeptide mimics[41] and boceprevir analogues with antiviral activity IC_{50} = 1 μM.[42] The covalent inhibitor halicin was also shown to be a nM M^{pro} inhibitor but was not tested against other proteases or for its antiviral activity against SARS-CoV-2.[43] An orally available low nM M^{pro} α-ketoamide containing inhibitor Y180 improved survival in a mouse model of SARS-CoV-2 infection.[44] Several early in vitro hits for M^{pro} when tested in the mouse model ultimately had modest efficacy. For example, GC376[14] is a potent inhibitor of SARS-CoV-2 M^{pro} (K_i = 12 nM)[45] with sub μM antiviral activity in A549+ACE2 cells and in Vero cells[45] but performed poorly in vivo.[46]

Compared to M^{pro}, there have been far fewer publications describing studies attempting to find PL^{pro} inhibitors. For example, one study identified disulfiram and analogues[47] as covalent inhibitors and another described the natural product celastrol,[48] which binds similarly to PL^{pro}, M^{pro}, and cathepsin L. Yet, another natural product, anarcardic acid, is a weak inhibitor of PL^{pro} and M^{pro}.[49] An initial hit for PL^{pro} came from the naphthalene derivative GRL-0617, which had previously been identified for SARS-CoV and demonstrated activity against SARS-CoV-2.[50] A study identified analogues of GRL-0617, but these did not have desirable antiviral activity versus SARS-CoV-2.[51] Another study described an analogue with sub μM PL^{pro} inhibition and antiviral activity.[52] Several virtual repurposing screens have used docking against PL^{pro} to identify hits such as the antimalarial mefloquine.[53] A combination of pharmacophores and docking led to 4 low μM hits against PL^{pro} although no antiviral activity was assessed.[54] A MedChemExpress library high throughput screen of 9791

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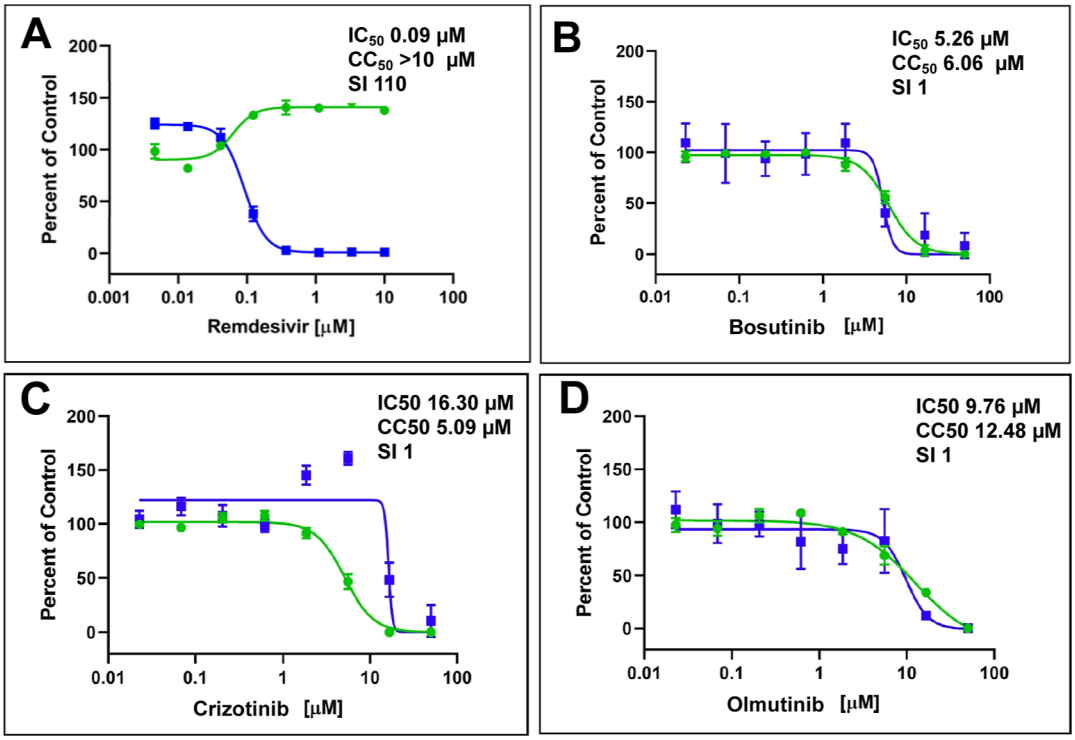

Figure 4. Kinase inhibitors tested against SARS-CoV-2 in Calu-3 cells. (A) Control: remdesivir, (B) bosutinib, (C) crizotinib, and (D) olmutinib. Sample well data was normalized to DMSO control wells and plotted versus drug concentration to determine the IC_{50} (infection: blue) and CC_{50} (toxicity: green). The *x* axis shows concentrations in a logarithmic scale.

compounds against PL^{pro} led to three hits including the clinical candidate FXR agonist tropifexor[55] which was a low μM hit active in Calu-3 cells. A docking and structure-based design approach was used to identify indole dual inhibitors for PL^{pro} and M^{pro} with antiviral activity.[56] We have previously described the in vitro (A549-ACE2 IC_{50} = 0.23 μM) and in vivo efficacy of pyronaridine tetraphosphate against SARS-CoV-2,[57] while more recently, it has been demonstrated to also inhibit PL^{pro} (IC_{50} = 1.8 μM).[58] We additionally described several pyronaridine analogues with similar activity as well as analogues that lacked activity altogether. Pyronaridine is an antimalarial drug that is approved in Europe and used as an antimalarial. It may hold promise as it has showed increased IFN-1β levels and decreased IL-6, CXCL1, and CCL4 while also decreasing viral load and improving lung histopathology in mice infected witih SARS-CoV-2.[58]

Herein, we have now described the screening of the Microsource Spectrum collection that led to the identification of cetylpyridinium chloride and raloxifene as PL^{pro} and M^{pro} inhibitors. We had earlier used a text mining approach to identify molecules with antiviral effects against coronaviruses[59] and this uncovered cetylpyridinium chloride, a quaternary ammonium compound which is widely used in mouthwashes, toothpastes, lozenges, throat sprays, breath sprays, and nasal sprays.[59] The target for this molecule against SARS-CoV-2 was previously unknown. Others have described the SARS-CoV-2 virucidal activity of this molecule in Vero cells upon short exposure and that it interfered with the spike and ACE2 interaction.[60] This antiviral efficacy seems also to be observed across different viral strains and in saliva, while the mechanism is occurring without disruption of the viral particles and that the denaturing effect of the spike protein may have a role.[61] Cetylpyridinium chloride has also been tested in Caco-2 cells (IC_{50} = 0.62 μM).[23] Several clinical trials have also indicated efficacy against SARS-CoV-2.[62,63] A lipidomics study has compared host and viral cell composition showing that the cholesterol/phospholipid ratio is comparable to lysosomes, as well as evaluated cetylyridinium chloride containing mouthwashes to show a viral reduction in vitro. This was followed by a clinical study, which showed that mouthwash containing cetylpyridinium chloride reduced the viral load.[24]

In contrast, raloxifene is a selective estrogen receptor modulator approved in the US and Europe for osteoporosis in post-menopausal women and reduces the potential risk of breast cancer.[64] A pseudovirus screen against MERS identified raloxifene, which also had activity against SARS-CoV-2 in Vero cells and in the hamster model reduced the virus in the lung.[21] Raloxifene was tested in Vero (IC_{50} = 5.9 μM) and Calu-3 cells (IC_{50} = 9 μM and SI 2.7) and was similarly active across different variants.[20] A screen of Sendai virus in human iPSCs also identified raloxifene, which was subsequently tested against SARS-CoV-2 in Vero cells (IC_{50} = 3.9 μM).[22] Raloxifene has also completed a Phase 2 randomized, double blinded, placebo-controlled trial in patients with early mild to moderate COVID-19 and showed evidence of effect in the primary endpoint and shortened the time for viral shedding.[65]

There have been relatively few studies that have to date described molecules that inhibit both M^{pro} and PL^{pro}; these include the covalent inhibitor disulfiram[66,67] and non-covalent HCV protease inhibitors grazoprevir, simeprevir, and vaniprevir.[68] A recent screen of over 1.8 million compounds found only six molecules that inhibited both M^{pro} and PL^{pro}.[69]

FDA-approved small-molecule kinase inhibitors could potentially be repurposed as antivirals because many kinase host targets are necessary or required for the viral life cycle, replication, and infection of multiple virus types.[70] We recently

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demonstrated that vandetanib blocks the cytokine storm in mice infected by SARS-CoV-2,[19] which prompted us to evaluate other kinase inhibitors against PL^{pro} and M^{pro}. Here, we have demonstrated for the first time that olmutinib, bosutinib, dacomitinib, and crizotinib are PL^{pro} inhibitors, and this may contribute alongside any host effects the compounds may have. Olmutinib showed the lowest IC_{50} = 0.54 ± 0.04 μM against PL^{pro} in this study, and it is also an investigational anti-cancer drug for non-small cell lung cancer (NSCLC), which inhibits the epidermal growth factor receptor (EGFR) by binding covalently to a cysteine residue near the kinase domain.[71] Whether olmutinib binds covalently remains to be investigated. Dacomitinib is a selective and irreversible inhibitor of EGFR, approved for the treatment of NSCLC with EGFR gene mutation,[72] which demonstrated potent antiviral activity against SARS-CoV-2 in Calu-3 cells with IC_{50} = 0.04 μM and CC_{50} = 9 μM.[73] EGFR is an essential pathway for SARS-CoV-2 replication[74] and many viruses such as hepatitis C, Epstein-Barr virus, and influenza, have been shown to use the EGFR as an entry receptor.[75−77] Bosutinib is a BCR-ABL and proto-oncogene tyrosine-protein kinase Src inhibitor used for the treatment of chronic myelogenous leukemia[78] and it was previously demonstrated to have *anti*-SARS-CoV-2 entry activity with an EC_{50} = 2.45 μM and an SI = 7.08 in Vero cells.[79] Crizotinib is an anaplastic lymphoma kinase (ALK) and an inhibitor used for the treatment of NSCLC.[80,81] Previously, it was shown that (*S*)-crizotinib inhibited SARS CoV-2 in three cell lines and variants of concern such as delta and omicron, while crizotinib hydrochloride showed activity in one cell line Vero-TMPRSS2.[82] Olmutinib, bosutinib, and crizotinib were tested by us in Calu-3 cells and demonstrated cell toxicity. The activity of these compounds in other cell lines such as A549-ACE2, Huh-7, and Caco-2 needs to be determined, as we and others have previously demonstrated cell-type specific activities of compounds against SARS-CoV-2.[57,73]

In conclusion, we have described the discovery of several new repurposed inhibitors for PL^{pro} and M^{pro} using high-throughput screening of a small library of drugs and the specific selection of kinase inhibitors that had previously demonstrated in vitro activity in cell lines, suggesting a direct antiviral effect. All these molecules are clinically accessible and could be further evaluated in animal models or clinical studies for SARS-CoV-2. Perhaps the molecule of most interest to us is cetylpyridinium chloride. While it does not represent an oral drug, its application as a mouthwash or nasal spray may have therapeutic applications, as demonstrated in several clinical studies, and this was also proposed by us at the very outset of the pandemic.[59] We have now described for the first time how PL^{pro} and M^{pro} may represent the viral targets for this readily available molecule that may have already been in widespread use during the pandemic in various consumer products.

METHODS

Chemicals and Reagents. The Microsource Spectrum Collection compound library was used for screening. Bosutinib, crizotinib, dacomitinib, cetylpyridinium chloride, and raloxifene were purchased from MedChemExpress (MCE). The purity of these compounds is greater than 95%. Compound 15c[83] was purchased from Sigma-Aldrich.

SARS-CoV-2 High-Throughput Screening against PL^{pro} and M^{pro}. SARS-Cov-2 PL^{pro} and M^{pro} expression, purification, and activity assays were described previously.[58] Briefly, the M^{pro} inhibition assay was performed using an fluorescence resonance energy transfer (FRET)-based fluorescent peptide substrate DABCYL-KTSAVLQ↓SGFRKM-E(EDANS)-NH2 (purchased from Genscript), with an enzyme concentration of 140 nM and a 30 μM fluorescent substrate in assay buffer (20 mM Tris pH 7.3, 1 mM EDTA, and 1 mM DTT) at 37 °C for 30 min. Activity was detected in the spectrofluorometer system Spectramax Gemini EM (Molecular Devices), with λ_{ex} = 360 nm and λ_{em} = 460 nm. For the PL^{pro}, a FRET-based fluorescent peptide substrate, Abz-TLKGG↓APIKEDDPS-EDDnp (kindly provided by Dr. Maria Aparecida Juliano, Federal University of São Paulo, Brazil), was used. For the reaction, compounds were incubated with 70 nM enzyme and 27 μM fluorescent substrate in PL^{pro} assay buffer (50 mM HEPES pH 7.5, 0.01% Triton X-100, and 5 mM DTT) at 37 °C for 30 min. Activity was measured in the plate reader system, Spectramax Gemini EM (Molecular Devices), with λ_{ex} = 320 nm and λ_{em} = 420 nm. For both, controls in reactions without enzyme (negative control) and without inhibitor (positive control).

The Microsource Spectrum collection library containing 2560 compounds was tested against PL^{pro} and M^{pro} in a high-throughput screening format (HTS) using 384 well-plates.

Proteins in the described assay conditions[58] were incubated with 1% DMSO and compounds at 10 μM for 30 min at 37° C in an end-point format. The activity of compound reaction was normalized based on the relative activity of the enzymes in the presence of 1% DMSO. Compounds that inhibited each protease activity in more than 80% were assayed in a dose-dependent manner to determine their half-inhibitory concentrations, IC_{50}. The results were analyzed using OriginPro 9.0 Software (Origin Lab), and the IC_{50} for each compound was estimated using the Hill1 function fitting. The dose response assays were carried out in triplicate of the same point in the experiment.

Calu-3 Cells. Compounds were incubated for 2 h with Calu-3 (ATCC, HTB-55) cells, followed by infection with SARS-CoV-2 (isolate USA WA1/2020) at a MOI = 0.5. 48 h post-infection, cells were fixed, immunostained, and imaged by automated microscopy for infection (dsRNA + cells/total cell number) and cell number. Sample well data was normalized to aggregated DMSO control wells and plotted versus drug concentration to determine the IC_{50} (infection: blue) and CC_{50} (toxicity: green).

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Author Contributions

All authors read and accept the manuscript. A.S.G., A.C.P., R.S.F., G.O., and S.E. conceived and codirected the study. A.C.P., A.S.G., and R.S.F. designed the experiments. A.S.G., G.D.N., A.M.N., and V.O.G. performed in vitro experiments. A.C.P. and S.E. drafted the manuscript.

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Notes

The authors declare the following competing financial interest(s): SE is CEO of Collaborations Pharmaceuticals, Inc. ACP is an employee at Collaborations Pharmaceuticals, Inc. Other authors have no conflicts.

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■ ABBREVIATIONS USED

ALK, anaplastic lymphoma kinase; COVID-19, coronavirus disease; EGFR, epidermal growth factor receptor; HIV, human immunodeficiency virus; HCV, hepatitis C virus; M^{pro}, main protease; MERS-CoV, middle east respiratory syndrome coronavirus; NSCLC, non-small cell lung cancer; PL^{pro}, papain-like protease; P-gp, P-glycoprotein; ROS1, c-ros oncogene 1; SARS-CoV-2, severe acute respiratory coronavirus 2; VGFR, vascular endothelial growth factor

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