Characterization of SARS-CoV main protease and identification of biologically active small molecule inhibitors using a continuous fluorescence-based assay

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Abstract Severe acute respiratory syndrome associated coronavirus main protease (SARS-CoV M\textsuperscript{pro}) has been proposed as a prime target for anti-SARS drug development. We have cloned and overexpressed the SARS-CoV M\textsuperscript{pro} in Escherichia coli, and purified the recombinant M\textsuperscript{pro} to homogeneity. The kinetic parameters of the recombinant SARS-CoV M\textsuperscript{pro} were characterized by high performance liquid chromatography-based assay and continuous fluorescence-based assay. Two novel small molecule inhibitors of the SARS-CoV M\textsuperscript{pro} were identified by high-throughput screening using an internally quenched fluorogenic substrate. The identified inhibitors have \(K_i\) values at low \(\mu\)M range with comparable anti-SARS-CoV activity in cell-based assays.

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Keywords: Severe acute respiratory syndrome; Coronavirus; 3C-like protease; Main protease; Fluorogenic substrate; Small molecule inhibitor

1. Introduction

Severe acute respiratory syndrome (SARS) swept through the world last year, infecting more than 8000 people across 29 countries and causing more than 900 fatalities [1]. The etiological agent of SARS was identified rapidly as a novel coronavirus of possible zoonotic origin [2–4]. Inadequate knowledge of the novel coronavirus SARS-CoV and the absence of efficacious therapeutics, however, were the main reasons for the failure to improve the outcome of the patients and to manage the outbreak of SARS effectively.

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Abbreviations: SARS-CoV, severe acute respiratory syndrome associated coronavirus; M\textsuperscript{pro}, main protease; HPLC, high performance liquid chromatography; HTS, high throughput screening; RT-PCR, reverse transcription polymerase chain reaction; PCR, polymerase chain reaction; IPTG, isopropyl \(\beta\)-D-thiogalactoside; DABCYL, 4-(4-dimethylaminophenyl)azo]benzoyl; EDANS, 5-(2-aminoethylamino)-1-naphthalenesulphonic acid; CPE, cytopathic effect; PRA, plaque reduction assay; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; PFU, plaque forming unit; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CMV, cytomegalovirus

Similar to other coronaviruses, SARS-CoV is an enveloped, positive-strand RNA virus with a large single-strand RNA genome comprised of \(\sim 29\,700\) nucleotides [5,6]. Among various open reading frames identified, the replicate gene encodes two overlapping polyproteins, pp1a and pp1ab, and comprises approximately two-thirds of the genome. Since the viral polyproteins are largely processed by the main protease (M\textsuperscript{pro}), and based on the successful development of efficacious antiviral agents targeting 3C-like proteases in other viruses, this “essential” protease is considered as a prime target for anti-SARS drug development [7,8]. In addition, the recently available crystal structure of the SARS-CoV M\textsuperscript{pro} has made possible the employment of structure-based drug design to develop M\textsuperscript{pro}-specific inhibitors [9].

To date, a number of potential inhibitors of SARS-CoV have been proposed using molecular modelling and virtual screening techniques [10–14]. However, the inhibitory activities of most of the proposed inhibitors have not yet been examined in in vitro assays employing purified SARS-CoV M\textsuperscript{pro} and synthetic substrates because of the tedious procedures involved in conventional high performance liquid chromatography (HPLC)-based cleavage assays. In addition, HPLC-based cleavage assays are impractical for high-throughput screening (HTS) of thousands of compounds from chemical libraries. A continuous fluorescence-based assay system will therefore be of great interest to the field for the rapid screening and evaluation of the potencies of potential inhibitors of SARS-CoV M\textsuperscript{pro}.

We have acquired a chemical library (ChemBridge Corporation) of 50,240 structurally diverse small molecule compounds that vary in functional groups and charges. A diverse chemical library was purposely chosen for anti-SARS drug screening, since we set out to isolate biologically active small molecules perturbing various viral components of the SARS-CoV in a cellular model of infection. A total of 104 compounds protected permissive Vero cells (African green monkey kidney cells) from SARS-CoV infection. Identification of inhibitors of SARS-CoV M\textsuperscript{pro} from this pool of compounds using conventional HPLC-based assays is labor-intensive and time consuming. Here, we report the enzymatic characterization of a recombinant SARS-CoV M\textsuperscript{pro} with authentic SARS-CoV M\textsuperscript{pro} amino acid sequence and the employment of a continuous fluorescence-based assay to identify novel small molecule inhibitors of SARS-CoV M\textsuperscript{pro}. The efficacies of the 2 selected...
inhibitors have also been evaluated in cell-based antiviral assays. Our study has defined the kinetic parameters of SARS-CoV M\textsuperscript{pro} in HPLC-based and continuous fluorescence-based assays and validated the usefulness of the fluorescence-based assay in HTS. Our results also provide biologically active novel non-peptide lead compounds for rational drug design of SARS-CoV M\textsuperscript{pro} inhibitors.

2. Materials and methods

2.1. Cloning of SARS-CoV M\textsuperscript{pro} and construction of plasmid pET

SARS-CoV (strain HKU39849) RNA was extracted from cell lysates of virus-infected Vero cells (African green monkey kidney cell line) by TRIZOL (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using ThermoScript RT-PCR system purchased from Invitrogen. The full-length cDNA was subsequently amplified by PCR using forward primer SVMPF (5'-CGGGGATCCGATCGAAGGTCGTAGTGGTTTTAGGAAA-ATG-3') and reverse primer SVMPR (5'-CGGAATTCTTATTTGGA-GTAA-GTACACCGA-3'). PCR product was separated by agarose gel electrophoresis, purified using QIAquick gel extraction kit (Qiagen), digested with BamHI and EcoRI restriction endonucleases, ligated to BamHI-EcoRI-digested PET28b DNA (Novagen), and transformed into E. coli DH5\textalpha cells by electroporation to generate pET SVMP. The nucleotide sequence of the SARS-CoV M\textsuperscript{pro} gene in plasmid pET SVMp was sequenced to confirm that no undesired mutation has been introduced. The construct was designed in a way that a factor Xa cleavage site was engineered in the N-terminal part of the SARS-CoV M\textsuperscript{pro} (His-Tag... EGER | SGFRKM...), the factor Xa cleavage site is indicated with a | and the released N-terminal part of the SARS-CoV M\textsuperscript{pro} is bolded). Cleavage with factor Xa released the His-tag and yields recombinant SARS-CoV M\textsuperscript{pro} with authentic SARS-CoV M\textsuperscript{pro} amino acid sequence. The identity of the purified SARS-CoV M\textsuperscript{pro} was determined by mass spectrometry (Genome Research Centre, the University of Hong Kong).

2.2. Protein expression and purification

Escherichia coli BL21 Gold (DE3) cells (Novagen) transformed with plasmid pET SVMp were grown at 37 °C with shaking in Luria-Bertani broth containing 50 μg/ml of kanamycin. The culture was induced with 0.5 mM of isopropyl β-D-thiogalactoside (IPTG) and grown at 30 °C with shaking for 4 h. Cells were harvested by centrifugation at 5000 × g at 4 °C for 20 min and disrupted by sonication in buffer A containing 20 mM Tris–HCl, pH 7.3, and 150 mM NaCl. Lysed cells were centrifuged at 12 000 × g at 4 °C for 20 min and the supernatant was decanted for further manipulation. The fusion SARS-CoV M\textsuperscript{pro} was purified by affinity purification using HiTrap TM Chelating chromatography using an excitation wavelength of 335 nm and emission wavelength of 495 nm (10 nm slit). Standard assay conditions were buffer A at 25 °C. Initial fluorescence was measured for substrate concentrations from 2.5 to 50 μM. To establish the linearity between enzyme concentration and rate of cleavage, the initial rate of change of fluorescence was measured at several SARS-CoV M\textsuperscript{pro} concentrations (100–800 nM) using 5 μM fluorogenic peptide as substrate. Assuming that the substrate concentration used was much lower than the K\textsubscript{m} of SARS-CoV M\textsuperscript{pro}, we determined the kinetic parameters and fluorescence properties of the substrate using the following equations:

\[
F_t = F_0 - (F_0 - F_e) e^{-(k_{cat}/K_m)t}
\]

(1)

\[
F_t = F_0 + (F_e - F_0) (k_m/K_m) t/\tau
\]

(2)

where \(F_t\) is the fluorescence intensity measured at a given time \(t\) during the reaction, \(F_0\) is the intensity of the substrate prior to the addition of enzyme, \(F_e\) is the intensity of the product when all the substrates were cleaved with sufficient M\textsuperscript{pro} within a period of 30 min, and [E] is the concentration of SARS-CoV M\textsuperscript{pro} used in the reaction. Values of \(k_{cat}/K_m\) were determined either by non-linear least squares regression analysis of all data using Eq. (1) or by linear least squares regression analysis of initial velocity data using Eq. (2).

2.3. Cell-based antiviral assays

SARS-CoV strain HKU39849 was isolated from a SARS patient in Hong Kong. The degree of protection offered by the test compounds against SARS-CoV infection was measured by Vero cell cytopathic effect (CPE) assay and plaque reduction assay (PRA). For CPE assay, Vero cells were seeded at 2 × 10\textsuperscript{4} cells per well (96-well microtitre plate) in complete Eagle's minimal essential medium (EMEM) (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Invitrogen) with or without the addition of test compounds. One hundred TCID\textsubscript{50} (50% tissue culture infectious dose) of SARS-CoV was added subsequently to each well. Assay plates were incubated at 37 °C in 5% CO\textsubscript{2} and CPE of the infected cells were recorded 96 h post infection using a Leica DMIL inverted microscope equipped with DC300F digital imaging system (Leica Microsystems). For PRA, one

were confirmed by mass spectrometry (Genome Research Centre, the University of Hong Kong).

2.4. Fluorescence-based kinetic analysis

A synthetic fluorogenic peptide DABCYL-SAVLQ | SGFRKMK (SP2) mimicking an autolytic cleavage site (the cleavage site by SARS-CoV M\textsuperscript{pro} is indicated with a |) was synthesized by SynPep Corporation. Cleavage of the fluorogenic peptide was monitored continuously by a F-4500 fluorescence spectrophotometer (Hitachi) using an excitation wavelength of 355 nm (10 nm slit) and emission wavelength of 495 nm (10 nm slit). Standard assay conditions were buffer A at 25 °C. Initial fluorescence was measured for substrate concentrations from 2.5 to 50 μM. To establish the linearity between enzyme concentration and rate of cleavage, the initial rate of change of fluorescence was measured at several SARS-CoV M\textsuperscript{pro} concentrations (100–800 nM) using 5 μM fluorogenic peptide as substrate. Assuming that the substrate concentration used was much lower than the K\textsubscript{m} of SARS-CoV M\textsuperscript{pro}, we determined the kinetic parameters and fluorescence properties of the substrate using the following equations:

\[
F_t = F_0 - (F_0 - F_e) e^{-(k_{cat}/K_m)t}
\]

(1)

\[
F_t = F_0 + (F_e - F_0) (k_m/K_m) t/\tau
\]

(2)
hundred plaque forming units (PFU) of SARS-CoV were added to individual wells of 24-well tissue culture plates (TPP) seeded with a confluent monolayer of Vero cells (1 × 10^5 cells per well) in EMEM with 1% FBS. Plates were incubated at 37 °C in 5% CO_2 for 1 h. One milliliter of overlay (1% low-melting agarose in EMEM with 1% FBS and appropriate concentrations of inhibitors) was added to each well after the media were aspirated. After 48 h of incubation at 37 °C in 5% CO_2, cells were fixed by adding 1 ml of 10% formaldehyde and the agarose plugs removed. Cells were stained with 0.5% crystal violet in 70% methanol and the viral plaques counted. Experiments were carried out in quadruplicate and dose response data were best fit to logistic equation in SigmaPlot 8.0 (SPSS). The cytotoxicity of the inhibitors was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Roche) according to the manufacturer’s instructions. All procedures involving manipulation of live SARS-CoV were carried out in a biological safety level 3 containment laboratory.

3. Results

3.1. Biosynthesis and purification of recombinant SARS-CoV M^pro

The His-tag recombinant SARS-CoV M^pro was successfully expressed in E. coli and the full length authentic SARS-CoV M^pro was purified to homogeneity after affinity chromatography, factor Xa cleavage, anion-exchange chromatography, and size-exclusion chromatography. The described protocol yields 10 mg of purified protein from 4 liters of culture. The employment of a synthetic substrate SP1 mimicking the putative autolytic cleavage site of the N-terminal part of M^pro in the HPLC-based cleavage assay established the specificity of the purified SARS-CoV M^pro (Fig. 1A). The turnover number of SARS-CoV M^pro and K_m on synthetic peptide SP1 was found to be 0.54 ± 0.04 s^{-1} and 2.3 ± 0.6 × 10^{-4} M, respectively, by Lineweaver–Burk plot and the k_{cat}/K_m was calculated to be 2.4 ± 0.6 × 10^5 M^{-1}s^{-1} (Fig. 1B).

3.2. Fluorescence-based kinetic analysis

The linearity of the initial fluorescence intensity gradually lost at concentrations above 10 μM of SP2 (Fig. 2A), a property very similar to a fluorogenic peptide designed for cytomegalovirus (CMV) 3C-like protease [15]. When 5 μM of SP2 was used, the initial rate of change of fluorescence intensity increased in a linear fashion with 100–800 nM of SARS-CoV M^pro (Fig. 2B), indicating the usefulness of using this fluorescence-based substrate to conduct kinetic studies under the specified conditions. Using 5 μM of SP2 and 100–600 nM of SARS-CoV M^pro, a k_{cat}/K_m value of 2.9 ± 0.2 × 10^4 M^{-1}s^{-1} was obtained by applying Eqs. (1) and (2).

3.3. HTS for inhibitors of SARS-CoV M^pro

The 104 compounds were screened at 20 μg/ml. The rate of change of fluorescence intensity was normalized to control in the absence of inhibitors and the results are shown in Fig. 3.
Ten compounds exhibited greater than 50% reduction in the rate of change of fluorescence intensity when compared to the control in the absence of inhibitors and were therefore selected for further evaluations. Some of the hits were expected to be false positives because of delivery errors, light scattering, or optical absorbance of test compounds. The final evaluation of inhibitors was to be performed with a rigorous HPLC-based cleavage assay that is not subject to these artifacts. Of the 10 compounds tested at a concentration of 20 µl/ml with 200 nM SARS-CoV Mpro and 200 µM SP1 using the HPLC-based assay, two exhibited >50% inhibitory effects on the Mpro and were therefore regarded as true inhibitors (data not shown). The two identified inhibitors were designated MP576 and MP521 and their chemical structures are shown in Fig. 4.

### 3.4. $K_i$ of inhibitors of SARS-CoV Mpro

To determine the values of $K_i$, the concentration of the inhibitors was converted to molar units for more precise comparison of their inhibitory activities. The $K_i$ values of MP576 and MP521 were calculated (applying Eq. (3)) to be 2.9 ± 0.3 and 11 ± 2 µM, respectively, using fluorogenic substrate SP2 (Table 1).

### 3.5. Cell-based antiviral assays

At 10 µg/ml concentration, both inhibitors MP576 and MP521 protected the monolayer of Vero cells from SARS-CoV induced CPE (Fig. 5), indicating the promising antiviral activities and the non-toxic properties of these two compounds at the concentration tested. In addition, MP576 and MP521 inhibited the SARS-CoV plaque formation in Vero cells in a concentration dependent manner with EC50 (median effective concentration) values of 7 ± 2 and 14 ± 4 µM, respectively (Table 1), demonstrating that the protective effects observed were indeed due to the presence of Mpro inhibitors. Furthermore, the TC50 (median toxic concentration) values of MP576 and MP521 were determined to be >50 µM (Table 1), indicating that these two compounds are not cytotoxic at their effective antiviral concentrations.

### 4. Discussion

We set out to clone and to characterize the SARS-CoV Mpro with the intention to develop an assaying system amendable for HTS operations for isolating potential drug leads targeting this “essential” component of the SARS-CoV. Since the additional amino acid sequence present in recombinant Mpro resulting from cloning procedures might have undesirable properties in in vitro or in vivo assaying systems, we engineered the recombinant SARS-CoV Mpro in a way that the final purified recombinant SARS-CoV Mpro will have amino acid sequence identical to that of the authentic SARS-CoV Mpro. After successive purifications employing affinity chromatography, factor Xa cleavage, anion-exchange chromatog-

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**Table 1**

| Compound | $K_i$ (µM) | EC<sub>50</sub> (µM) | TC<sub>50</sub> (µM) |
|----------|------------|---------------------|---------------------|
| MP576    | 2.9 ± 0.3  | 7 ± 2               | >50                 |
| MP521    | 11 ± 2     | 14 ± 4              | >50                 |
raphy, and size-exclusion chromatography, we obtained a purified SARS-CoV M\(^{\text{pro}}\) with a \(k_{\text{cat}}/K_m\) value of \(2.4 \times 10^3\) M\(^{-1}\)s\(^{-1}\) using synthetic substrate SP1 and a \(k_{\text{cat}}/K_m\) value of \(2.9 \times 10^4\) M\(^{-1}\)s\(^{-1}\) using fluorogenic substrate SP2. The kinetic parameters obtained were comparable to that of other viral 3C-like proteases reported [16,17]. We noticed that the \(k_{\text{cat}}/K_m\) value (calculated to be \(1.8 \times 10^2\) M\(^{-1}\)s\(^{-1}\)) published by Lai’s group on a synthetic peptide substrate (H\(_2\)N-TSAVLQSGFRK-COOH) [18,19] is about 10-fold lower than the one we obtained with our synthetic substrate SP1 (H\(_2\)N-TSAVLQSGFRKW-COOH; \(k_{\text{cat}}/K_m\) value of \(2.4 \times 10^3\) M\(^{-1}\)s\(^{-1}\)).

Aside from differences in purification procedures and assaying conditions, and slight difference in amino acid sequence between the two peptides, we do not have an explanation for the apparent discrepancy between the results obtained by the two groups. The fluorogenic substrate SP2 used in the study is very sensitive for assaying the cleavage activity of the SARS-CoV M\(^{\text{pro}}\). As little as 6.5 nM of the SARS-CoV M\(^{\text{pro}}\) could be detected in the assay system we employed (data not shown). This ultra-sensitive substrate, however, could not be used at concentrations higher than 10 \(\mu\)M due to its internal quenching effects [15]; this property rendered SP2 unsuitable for the determination of \(K_m\) of the assay system. Nevertheless, SP2 has been demonstrated to be an excellent substrate for HTS purposes and for evaluation of inhibitor potencies.

The two small molecule compounds identified in our study are novel non-peptide inhibitors of SARS-CoV M\(^{\text{pro}}\). The fact that they inhibited the SARS-CoV M\(^{\text{pro}}\) with \(K_i\) values around 10 \(\mu\)M protected Vero cells from viral infection at comparable concentrations and exhibited low cytotoxicity towards Vero cells making them promising leads for anti-SARS drug development. Further investigations using analogues (CB5751 and CB5173) of these two SARS-CoV M\(^{\text{pro}}\) inhibitors suggested that the position of the nitro group in both inhibitors contributes substantially to their inhibitory activities: changing the 2-nitro group to 3-nitro group (Fig. 4C, D) readily reduced the compounds’ ability to inhibit the SARS-CoV M\(^{\text{pro}}\) (Fig. 6). We speculate that the 2-nitro group may be involved in forming productive bonding in the inhibitor-enzyme complex. Further structure-activity relationship studies employing more analogues of the identified compounds and site-directed mutagenesis with the SARS-CoV M\(^{\text{pro}}\) will help us to elucidate the mode of actions of these two novel inhibitors. While this manuscript is in preparation, a number of groups reported the cloning and production of different versions of the SARS-CoV M\(^{\text{pro}}\) [20,21]. Others described the development of in vitro assays for screening SARS-CoV M\(^{\text{pro}}\) inhibitors [22,23]. We report here the detailed kinetic analysis of a recombinant SARS-CoV M\(^{\text{pro}}\) with amino acid sequence identical to that of the authentic SARS-CoV M\(^{\text{pro}}\) and the successful identification of potent small molecule inhibitors of the M\(^{\text{pro}}\) with demonstrated anti-SARS-CoV activities in cellular models. With the establishment of assaying systems to examine the in vitro cleavage and the cellular anti-SARS-CoV activities, we can now start lead optimization by using focused combinatorial chemical libraries to understand the structure-activities relationship of the two leads and to yield compounds with far superior antiviral activities.

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