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Christopher J. Schofield *et al.*
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 Tika R. Malla, ^a Anthony Tumber,^a Tobias John, ^a Lennart Brewitz, ^a Claire Strain-Damerell, ^{bc} C David Owen, ^{bc} Petra Lukacik, ^{bc} H. T. Henry Chan, ^a Pratheesh Maheswaran, ^a Eidarus Salah,^a Fernanda Duarte, ^a Haitao Yang,^d Zihe Rao,^d Martin A. Walsh ^{bc} and Christopher J. Schofield ^{*a}

The main viral protease (M^{Pro}) of SARS-CoV-2 is a nucleophilic cysteine hydrolase and a current target for anti-viral chemotherapy. We describe a high-throughput solid phase extraction coupled to mass spectrometry M^{Pro} assay. The results reveal some β -lactams, including penicillin esters, are active site reacting M^{Pro} inhibitors, thus highlighting the potential of acylating agents for M^{Pro} inhibition.

The main viral protease (M^{Pro}) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)¹ is a COVID-19 treatment target.² M^{Pro} along with the papain-like protease (PL^{Pro}), processes initially translated viral polyproteins to give cleaved proteins with biological functions essential for viral replication in cells.³ Following formation of a non-covalent enzyme-substrate complex, M^{Pro} catalysis proceeds *via* His-41 enabled reaction of Cys-145 with a scissile peptide bond forming a hydrolytically labile thioester. M^{Pro} cleaves after glutamine-residues with a preference for small-residues on the C-terminal side of the cleaved amide (Fig. 1A and B).⁴

Most reported M^{Pro} assays measure fluorescence, as preceded for other protease assays.^{1,2b,5} Whilst efficient, such methods do not simultaneously monitor substrate depletion/product formation and some compounds interfere with fluorescence.⁶ We were thus interested in establishing an alternative mass spectrometry (MS)-based high-throughput M^{Pro} assay for identifying new inhibitors and testing known drugs.

Solid phase extraction coupled with MS (SPE-MS) has been applied to high-throughput screens of enzymes.⁷ We envisaged

it could simultaneously monitor both M^{Pro} substrate depletion/product formation and covalent modification. The latter is of interest because many reported inhibitors of nucleophilic cysteine enzymes work by covalent reaction.⁸ Here we report how such an assay enabled identification of new M^{Pro} inhibitors, including β -lactams, the most important antibacterial class.⁹

We developed conditions for an SPE-MS based SARS-CoV-2 M^{Pro} assay (0.15 μ M M^{Pro}, 2.0 μ M TSAVLQ/SGFRK-NH₂, 20 mM HEPES, pH 7.5, 50 mM NaCl) using protein prepared as reported.^{1,10} Isolated M^{Pro} was found to be active when monitoring turnover of peptide substrates, including TSAVLQ/SGFRK-NH₂ which was cleaved to give TSAVLQ and SGFRK-NH₂ fragments (Fig. 1C and D). Kinetic parameters were determined for the 11-mer substrate ($K_m = 14.4 \mu$ M; $k_{cat} = 2.7 \text{ min}^{-1}$), both by monitoring substrate depletion and N-terminal product fragment formation (Fig. S1, ESI†). The efficiency (k_{cat}/K_m) of M^{Pro} determined by SPE-MS (28 500 M⁻¹ s⁻¹) is comparable to that observed for a similar substrate Mca-AVLQ/SGFRK(Dnp)K using a fluorescence resonance energy transfer (FRET) assay (27 000 M⁻¹ s⁻¹, as reported and in our hands).¹ Steady state kinetics for a 37-mer substrate were also investigated; a 2-fold increase in k_{cat}/K_m (60 026 M⁻¹ s⁻¹) was observed (Fig. S2, ESI†). Comparison of kinetic parameters for the SARS-CoV-2 M^{Pro} and the related SARS-CoV M^{Pro} reveal similar k_{cat}/K_m values (though the values for SARS-CoV were somewhat lower when using shorter substrates in an HPLC assay) (Table S1, ESI†). Note, the interconversion between monomeric/dimeric forms of M^{Pro} has the potential to introduce complexity in kinetic analyses.¹¹

Next, the SPE-MS assay was validated for inhibition studies with ebselen,¹ N3,¹ disulfiram,¹ and boceprevir¹² using the 11-mer TSAVLQ/SGFRK-NH₂ substrate (Table 1 and Fig. S3, ESI†). The ebselen IC₅₀ was $\sim 0.09 \mu$ M under standard conditions (0.15 μ M M^{Pro}, 2.0 μ M TSAVLQ/SGFRK-NH₂ *i.e.* [S] < K_m , 20 mM HEPES, pH 7.5, 50 mM NaCl at ambient temperature) compared to an IC₅₀ of $\sim 0.67 \mu$ M¹ using a FRET assay (0.2 μ M M^{Pro}, 20 μ M Mca-AVLQ/SGFRK(Dnp)K *i.e.* [S] $\approx K_m$, 50 mM Tris-HCl, pH 7.3, 1 mM EDTA, 30 °C)¹ (Table 1, entry 1; Fig. S3D, ESI†).

^a Chemistry Research Laboratory, Department of Chemistry, 12 Mansfield Road, Oxford, OX1 3TA, UK. E-mail: christopher.schofield@chem.ox.ac.uk

^b Diamond Light Source, Harwell Science & Innovation Campus, Didcot, Oxfordshire OX11 0DE, UK

^c Research Complex at Harwell, Harwell Science & Innovation Campus, Didcot, Oxfordshire OX11 0FA, UK

^d Shanghai Institute for Advanced Immunochemical Studies and School of Life Science and Technology, ShanghaiTech University, Shanghai, China

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Fig. 1 SPE-MS assay monitoring M^{PrO} catalyzed cleavage of the TSAVLQ/SGFRK-NH₂ substrate. (A) M^{PrO} catalyzed hydrolysis of TSAVLQ/SGFRK-NH₂. (B) View from a structure of Cys-145 linked M^{PrO} -N3 complex (PDB ID: 6LU7),¹ the Cys-His dyad is in pink; substrate binding sites are labelled in blue. (C) Deconvoluted mass spectrum of substrate/cleaved products after 10 minute incubation with M^{PrO} . Note, the C-terminal product was not efficiently retained by the SPE cartridge resulting in a low abundance compared to the N-terminal cleavage product. Sodium ion adducts (+23 Da) for the TSAVLQ product (639 Da) and substrate (1214 Da) are labelled with magenta and green asterisks, respectively. (D) % substrate turnover based on integration of the total abundance of cleaved products (TSAVLQ or SGFRK-NH₂) or the individual products. Conditions: 0.15 μ M M^{PrO} , 2.0 μ M TSAVLQ/SGFRK-NH₂ (1192 Da) (20 mM HEPES, pH 7.5, 50 mM NaCl).

Table 1 IC_{50} s of selected M^{PrO} inhibitors determined using SPE-MS assays compared to those obtained using FRET assays

Inhibitor	IC_{50} (SPE-MS) [μ M] ^{ab}	IC_{50} (SPE-MS) ^{ac} [μ M]	IC_{50} (FRET) [μ M]
Ebselen	0.09 \pm 0.07	0.09 \pm 0.07	0.67 \pm 0.09 ¹
N3	0.04 \pm 0.01	0.03 \pm 0.01	n.d.
Disulfiram	0.60 \pm 0.01	0.46 \pm 0.02	9.35 \pm 0.18 ¹
Boceprevir	11.0 \pm 4.8	9.2 \pm 5.5	2.70 \pm 0.05 ¹²

^a Mean of two independent replicates each performed in technical duplicate ($n = 2 \pm$ standard deviation, SD). Conditions: 0.15 μ M M^{PrO} and 2.0 μ M TSAVLQ/SGFRK-NH₂ substrate in 20 mM HEPES, pH 7.5, 50 mM NaCl. ^b 30 min inhibitor preincubation. ^c 60 min inhibitor preincubation.

We optimized the assay for studying covalent modifications with a higher M^{PrO} concentration being used to enable robust analyses (1 μ M M^{PrO}), though IC_{50} and preliminary covalent modification data can be accumulated from the same experiment. SPE purification is denaturing, so monomer modification was observed. Assay validation used N3;¹ predominantly (but not exclusively) a single N3 adduct was observed (Fig. 2A, B and Fig. S4, ESI[†]), consistent with structural work revealing Cys-145 reaction¹ (Fig. 1B and Fig. S5, Table S2, ESI[†]). We exploited selective reaction of N3 to test selectivity of other inhibitors as exemplified with ebselen, comparing results for N3 treated/untreated M^{PrO} (Fig. 2C, E and Fig. S6, ESI[†]). By contrast with N3, we saw time dependent modification of multiple residues with ebselen with or without N3 pre-treatment (Fig. 2C and E), implying reaction of some of the 11 non-active site cysteines (Fig. S5, ESI[†]). Ebselen was used as a readily available positive inhibition control in subsequent studies.

The assay was used to screen the Library of Pharmacologically Active Compounds (LOPAC) and a library of 1600 small-molecule active pharmaceutical ingredients (API) at 20 μ M

compound (Fig. S7, ESI[†]). Excellent Z' -factors¹³ and signal to noise ratios reveal the assay robustness (Fig. S7, ESI[†]). In addition to ebselen (identification of which validates the method), diverse inhibitors ($\geq 80\%$ at a fixed 20 μ M inhibitor concentration) were identified, some (related to) known inhibitors,^{1,14} including auranofin, cisplatin, IPA-3, bismuth subsalicylate, thioguanine, carmustine, and disulfiram (Tables S3 and S4, ESI[†]).

IC_{50} s were determined for compounds with $\geq 80\%$ inhibition at 20 μ M, excluding known interference compounds.¹⁵ Auranofin ($IC_{50} \sim 1.5 \mu$ M; reported $IC_{50} \sim 0.5 \mu$ M¹⁴), an α -chloro ketone (TPCK) ($IC_{50} \sim 0.8 \mu$ M), IPA-3 ($IC_{50} \sim 0.1 \mu$ M), and 5-thioguanine ($IC_{50} \sim 13.5 \mu$ M) are some of the more potent inhibitors (Fig. S8, ESI[†]). Some of these covalently modified M^{PrO} , sometimes with more than one reaction being observed (Fig. S9–S15, ESI[†]). Active site selectivity was investigated using N3 treated and untreated M^{PrO} . Following N3 treatment, in some cases, *e.g.* TPCK and *N*_x-*p*-toluenesulfonyl-*L*-lysine chloromethyl ketone, substantial covalent modification was no longer observed, implying selective Cys-145 reaction (Fig. 2D, F and Fig. S12, S13, ESI[†]). Although further validation is required, with BAY 11-7082 and IPA-3 the multiple adducts observed with unmodified M^{PrO} were diminished when the active site was N3 blocked, suggesting reaction with Cys-145 might alter the M^{PrO} conformation (Fig. S9 and S11, ESI[†]).

The screen identified β -lactam drugs as potential M^{PrO} inhibitors, including penicillins and cephalosporins (Table S5, ESI[†]). This was of interest, as in preliminary work we observed some β -lactams react covalently (data not shown). β -Lactam antibiotics form stable acyl-enzyme complexes with bacterial nucleophilic serine enzymes; they inhibit other nucleophilic serine enzymes including proteases and β -lactamases¹⁶ and nucleophilic cysteine enzymes.¹⁷

Studies on cephalosporins identified as potential inhibitors from the screen revealed no substantial covalent M^{PrO} modification,





Fig. 2 N3 dependent reaction monitoring the active site selectivity of inhibitors. (A) Reaction of N3 with the M^{pro} active site Cys-145.¹ (B) N3, (C) ebselen and (D) TPCK modifies M^{pro} in a covalent manner. (E) Ebselen covalently modifies multiple M^{pro} cysteine residues in the presence of N3. (F) TPCK does not covalently modify M^{pro} in the presence of N3, suggesting that it selectively reacts with active site Cys-145. Black spectra: wild-type M^{pro} (33796 Da).

though cephalosporin C Zn(II) salt and cephalosporin C Na(I) salt inhibited. However, the IC₅₀s for cephalosporin C Zn(II) salt and ZnCl₂ were similar, indicating much of the inhibition is due to Zn(II) ions (Fig. S16, ESI[†]), as observed for cephalosporin C Zn(II) salt inhibition of other enzymes.^{7b}

We further investigated β -lactam reactions with M^{pro} using a diverse set of β -lactams (Fig. S17, ESI[†]). Though most β -lactams were inactive (IC₅₀ > 100 μ M), two penicillin esters manifested IC₅₀s < 5 μ M, *i.e.* 1: a penicillin V sulfone C3 benzyl ester (IC₅₀ ~ 1.5 μ M), and 2: a derivative of penicillin G sulfoxide C3

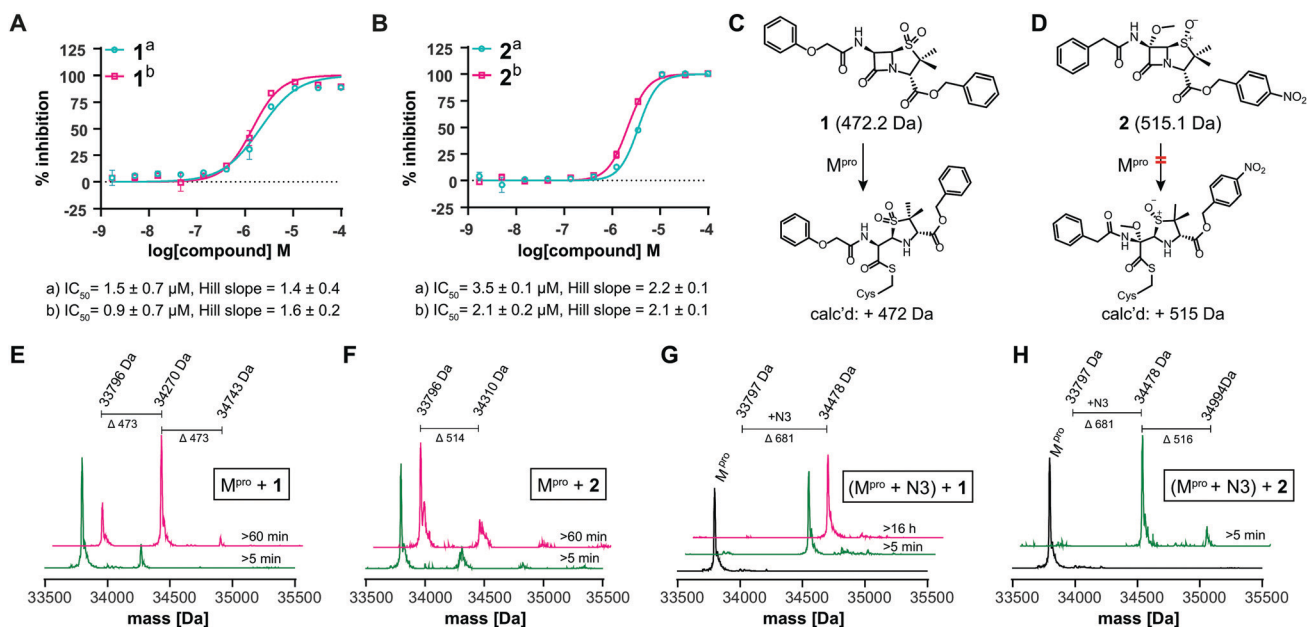


Fig. 3 β -Lactams inhibit M^{pro}. IC₅₀s for (A) 1 (penicillin V sulfone C3 benzyl ester) and (B) 2 (C6-methoxy penicillin G sulfoxide C3 p-nitrobenzyl ester) determined using SPE-MS; data are a mean of technical duplicates with (a) 30 min and (b) 60 min preincubation. IC₅₀s are means of two independent repeats each composed of technical duplicates ($n = 2 \pm SD$). Proposed reaction of 1 (C) and 2 (D) with M^{pro}. (E) A single molecule 1 covalently modifies M^{pro}. (F) 2 does not efficiently modify M^{pro} through covalent reaction. Conditions: 1 μ M M^{pro}, 20 μ M β -lactam, 20 mM HEPES, pH 7.5, 50 mM NaCl. (G) 1 does not covalently modify M^{pro} preincubated with N3, suggesting 1 reacts with Cys-145. (H) 2 does not efficiently react with M^{pro} preincubated N3. Conditions: 1 μ M M^{pro} preincubated with 3 μ M N3, 20 μ M 1 or 2, 20 mM HEPES, pH 7.5, 50 mM NaCl. Black spectra: wild-type M^{pro} (33796 Da).



p-nitrobenzyl ester ($IC_{50} \sim 3.5 \mu\text{M}$), both with similar potency with either 30 or 60 min preincubation (Fig. 3A and B). Other β -lactams inhibited, though more weakly (Fig. S17, ESI[†]). The inhibition by the penicillin benzyl esters may, in part, reflect binding of the N3 benzyl ester, likely binding in the P1' or P2 pocket (Fig. 1B).¹ Structures of M^{PRO} complexed with a β -lactam were not obtained; however, docking studies reveal potential of **1** and **2** to bind favourably at the active site (Fig. S18, ESI[†]), in the case of **1** in a manner enabling Cys-145 reaction.

Evidence for covalent reaction was observed with representatives of the penem, carbapenem prodrug, penicillin, penicillin sulfone, clavam, cephem, and monobactam β -lactam sub-families (Fig. S19–S21, ESI[†]). In some cases, *e.g.* clavulanate (Fig. S19H, ESI[†]) and moxalactam (Fig. S21F, ESI[†]), (partial) inhibitor fragmentation was observed. There was no clear correlation between a propensity to react covalently and M^{PRO} inhibition; in some cases evidence for partial covalent modification, but no inhibition was observed (Fig. S17, ESI[†]). Covalent modification was observed with **1**, but only to a small (<10%) extent with **2**, suggesting the latter likely inhibits principally by a non-covalent interaction (Fig. 3C–H). After Cys-145 blocking with N3, no reaction with **1** was observed (Fig. 3G). Minor further modification of Cys-145 reacted M^{PRO} was observed with **2** (Fig. 3H), suggesting the low levels of covalent modification by **2** do not solely involve Cys-145.

In summary, SPE-MS is a useful method for M^{PRO} assays enabling analysis of inhibition by both substrate depletion/product formation. The method complements reported *in vitro* M^{PRO} assays and compares favourably to those in terms of its robustness and ability to enable efficient high-throughput screening/repurposing efforts. The SPE-MS assay also enables ready analysis of covalent M^{PRO} modification and use of M^{PRO} reacted with a selective inhibitor such as N3/TPCK informs on whether covalent reaction of a test inhibitor occur at the active site or not.

Although the available evidence implies that β -lactams can inhibit M^{PRO} non-covalently, the observation that some react with and inhibit M^{PRO} by covalent active site modification should promote interest in the development of inhibitors for M^{PRO} and other thiol proteases working *via* cysteinyl *S*-acylation. By contrast with *S*-alkylating inhibitors, which can have toxicity issues, *S*-acylation has not been widely explored for nucleophilic cysteine proteases.

The identification of β -lactam containing M^{PRO} inhibitors with structures closely related to drugs should promote work on the development of related compounds for progression towards clinical use for treatment of COVID-19 and viral diseases.

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Conflicts of interest

There are no conflicts to declare.

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