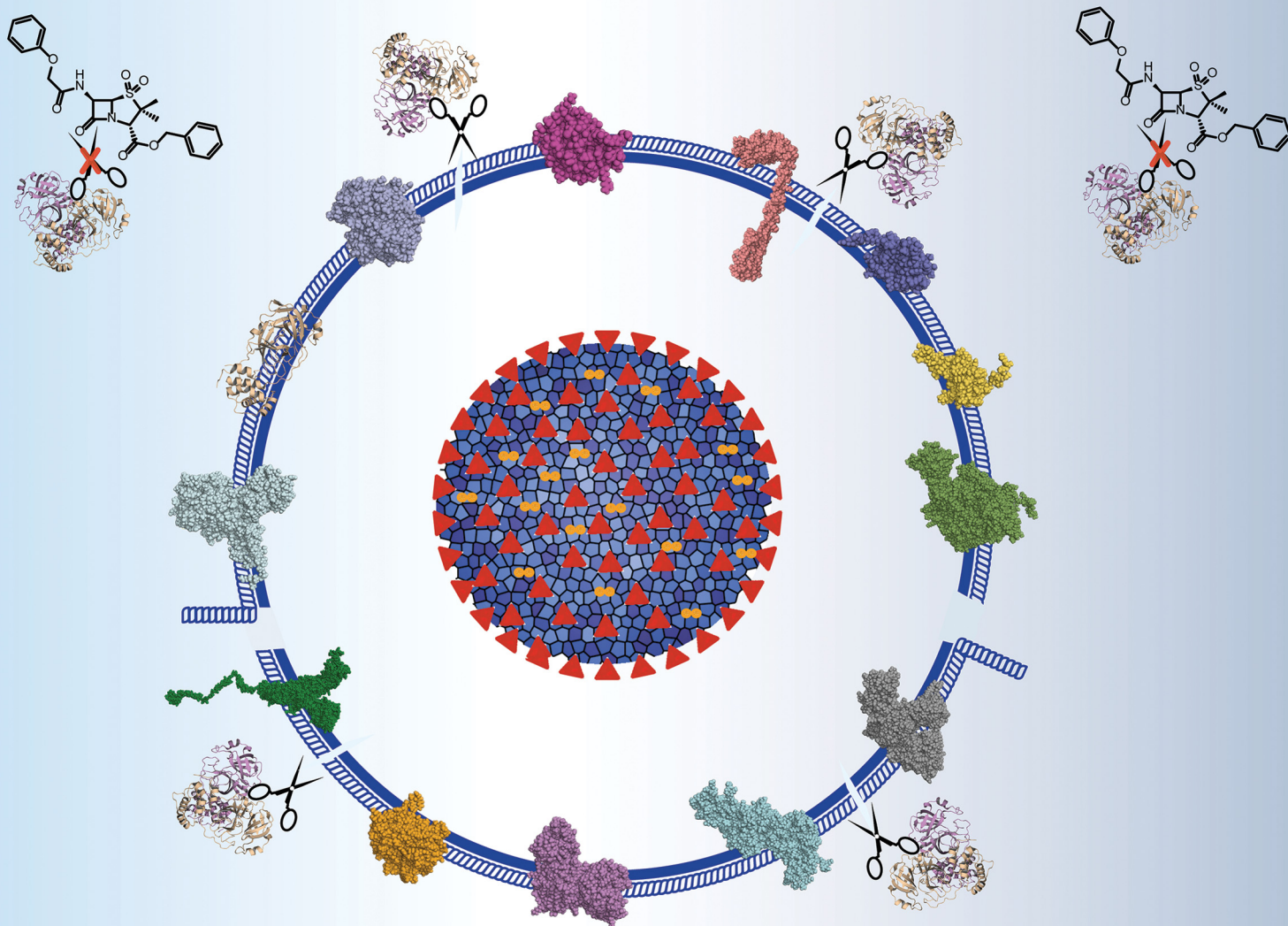


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# Mass spectrometry reveals potential of $\beta$ -lactams as SARS-CoV-2 M<sup>Pro</sup> inhibitors†

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**The main viral protease (M<sup>Pro</sup>) 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<sup>Pro</sup> assay. The results reveal some  $\beta$ -lactams, including penicillin esters, are active site reacting M<sup>Pro</sup> inhibitors, thus highlighting the potential of acylating agents for M<sup>Pro</sup> inhibition.**

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

Most reported M<sup>Pro</sup> assays measure fluorescence, as preceded for other protease assays.<sup>1,2b,5</sup> Whilst efficient, such methods do not simultaneously monitor substrate depletion/product formation and some compounds interfere with fluorescence.<sup>6</sup> We were thus interested in establishing an alternative mass spectrometry (MS)-based high-throughput M<sup>Pro</sup> 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.<sup>7</sup> We envisaged

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

We developed conditions for an SPE-MS based SARS-CoV-2 M<sup>Pro</sup> assay (0.15  $\mu$ M M<sup>Pro</sup>, 2.0  $\mu$ M TSAVLQ/SGFRK-NH<sub>2</sub>, 20 mM HEPES, pH 7.5, 50 mM NaCl) using protein prepared as reported.<sup>1,10</sup> Isolated M<sup>Pro</sup> was found to be active when monitoring turnover of peptide substrates, including TSAVLQ/SGFRK-NH<sub>2</sub> which was cleaved to give TSAVLQ and SGFRK-NH<sub>2</sub> 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<sup>Pro</sup> determined by SPE-MS (28 500 M<sup>-1</sup> s<sup>-1</sup>) 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<sup>-1</sup> s<sup>-1</sup>, as reported and in our hands).<sup>1</sup> Steady state kinetics for a 37-mer substrate were also investigated; a 2-fold increase in  $k_{cat}/K_m$  (60 026 M<sup>-1</sup> s<sup>-1</sup>) was observed (Fig. S2, ESI†). Comparison of kinetic parameters for the SARS-CoV-2 M<sup>Pro</sup> and the related SARS-CoV M<sup>Pro</sup> 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<sup>Pro</sup> has the potential to introduce complexity in kinetic analyses.<sup>11</sup>

Next, the SPE-MS assay was validated for inhibition studies with ebselen,<sup>1</sup> N3,<sup>1</sup> disulfiram,<sup>1</sup> and boceprevir<sup>12</sup> using the 11-mer TSAVLQ/SGFRK-NH<sub>2</sub> substrate (Table 1 and Fig. S3, ESI†). The ebselen IC<sub>50</sub> was  $\sim 0.09 \mu$ M under standard conditions (0.15  $\mu$ M M<sup>Pro</sup>, 2.0  $\mu$ M TSAVLQ/SGFRK-NH<sub>2</sub> *i.e.* [S] <  $K_m$ , 20 mM HEPES, pH 7.5, 50 mM NaCl at ambient temperature) compared to an IC<sub>50</sub> of  $\sim 0.67 \mu$ M<sup>1</sup> using a FRET assay (0.2  $\mu$ M M<sup>Pro</sup>, 20  $\mu$ M Mca-AVLQ/SGFRK(Dnp)K *i.e.* [S]  $\approx K_m$ , 50 mM Tris-HCl, pH 7.3, 1 mM EDTA, 30 °C)<sup>1</sup> (Table 1, entry 1; Fig. S3D, ESI†).

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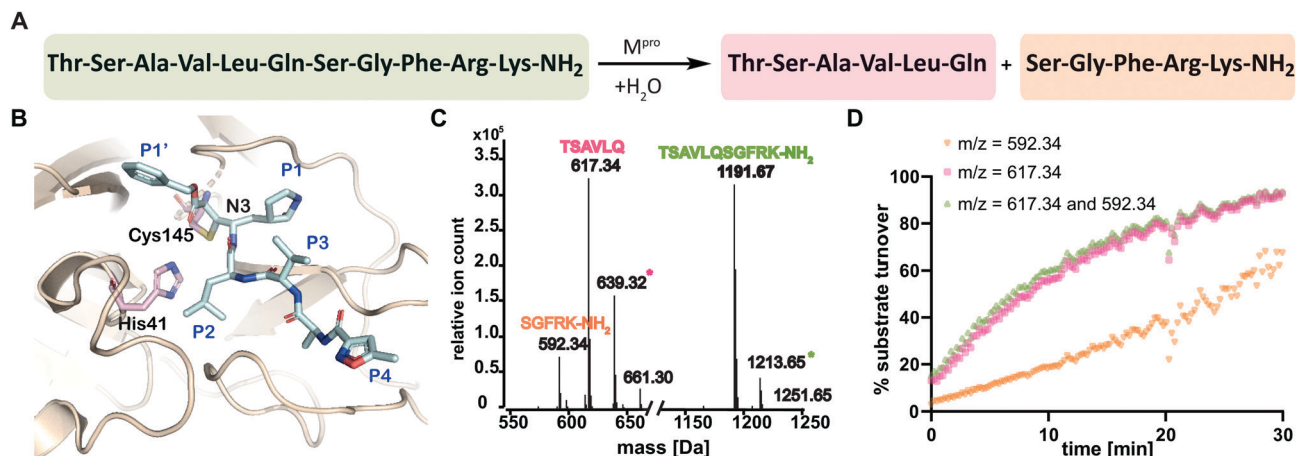
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**Fig. 1** SPE-MS assay monitoring  $M^{PrO}$  catalyzed cleavage of the TSAVLQ/SGFRK-NH<sub>2</sub> substrate. (A)  $M^{PrO}$  catalyzed hydrolysis of TSAVLQ/SGFRK-NH<sub>2</sub>. (B) View from a structure of Cys-145 linked  $M^{PrO}$ -N3 complex (PDB ID: 6LU7),<sup>1</sup> 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-NH<sub>2</sub>) or the individual products. Conditions: 0.15  $\mu$ M  $M^{PrO}$ , 2.0  $\mu$ M TSAVLQ/SGFRK-NH<sub>2</sub> (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) [ $\mu$ M] <sup>ab</sup>	$IC_{50}$ (SPE-MS) <sup>ac</sup> [ $\mu$ M]	$IC_{50}$ (FRET) [ $\mu$ M]
Ebselen	0.09 $\pm$ 0.07	0.09 $\pm$ 0.07	0.67 $\pm$ 0.09 <sup>1</sup>
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 <sup>1</sup>
Boceprevir	11.0 $\pm$ 4.8	9.2 $\pm$ 5.5	2.70 $\pm$ 0.05 <sup>12</sup>

<sup>a</sup> Mean of two independent replicates each performed in technical duplicate ( $n = 2 \pm$  standard deviation, SD). Conditions: 0.15  $\mu$ M  $M^{PrO}$  and 2.0  $\mu$ M TSAVLQ/SGFRK-NH<sub>2</sub> substrate in 20 mM HEPES, pH 7.5, 50 mM NaCl. <sup>b</sup> 30 min inhibitor preincubation. <sup>c</sup> 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  $\mu$ 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;<sup>1</sup> predominantly (but not exclusively) a single N3 adduct was observed (Fig. 2A, B and Fig. S4, ESI<sup>†</sup>), consistent with structural work revealing Cys-145 reaction<sup>1</sup> (Fig. 1B and Fig. S5, Table S2, ESI<sup>†</sup>). 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<sup>†</sup>). 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<sup>†</sup>). 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  $\mu$ M

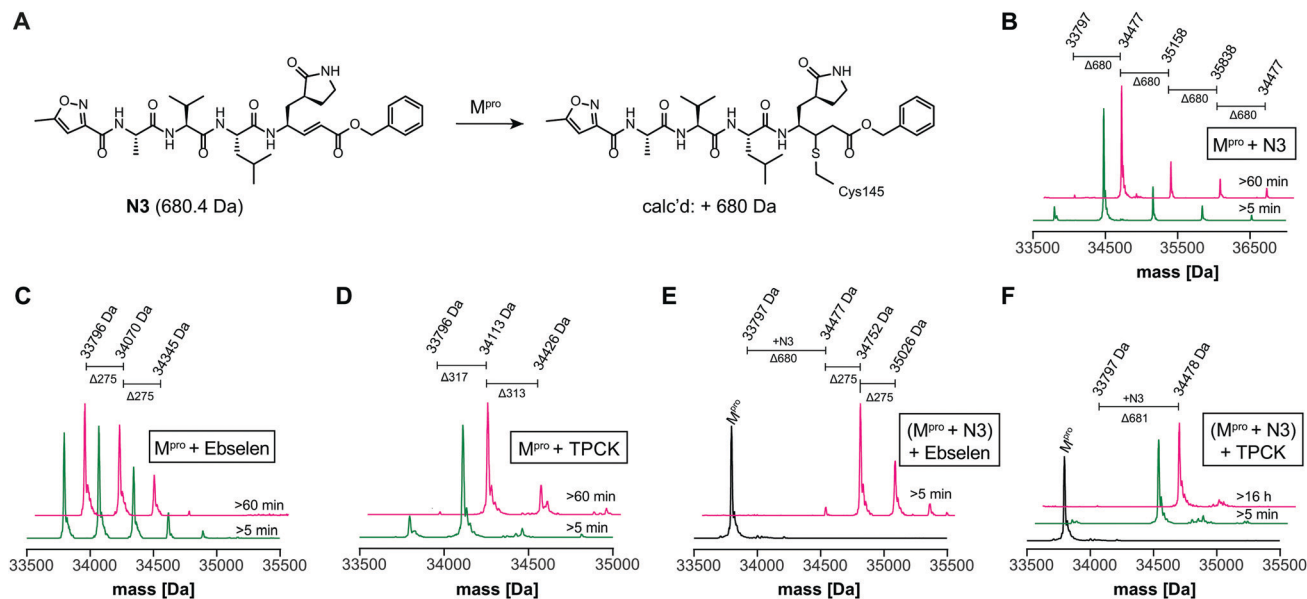
compound (Fig. S7, ESI<sup>†</sup>). Excellent Z'-factors<sup>13</sup> and signal to noise ratios reveal the assay robustness (Fig. S7, ESI<sup>†</sup>). In addition to ebselen (identification of which validates the method), diverse inhibitors ( $\geq 80\%$  at a fixed 20  $\mu$ M inhibitor concentration) were identified, some (related to) known inhibitors,<sup>1,14</sup> including auranofin, cisplatin, IPA-3, bismuth subsalicylate, thioguanine, carmustine, and disulfiram (Tables S3 and S4, ESI<sup>†</sup>).

$IC_{50}$ s were determined for compounds with  $\geq 80\%$  inhibition at 20  $\mu$ M, excluding known interference compounds.<sup>15</sup> Auranofin ( $IC_{50} \sim 1.5 \mu$ M; reported  $IC_{50} \sim 0.5 \mu$ M<sup>14</sup>), an  $\alpha$ -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<sup>†</sup>). Some of these covalently modified  $M^{PrO}$ , sometimes with more than one reaction being observed (Fig. S9–S15, ESI<sup>†</sup>). Active site selectivity was investigated using N3 treated and untreated  $M^{PrO}$ . Following N3 treatment, in some cases, e.g. TPCK and N<sub>x</sub>-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<sup>†</sup>). 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<sup>†</sup>).

The screen identified  $\beta$ -lactam drugs as potential  $M^{PrO}$  inhibitors, including penicillins and cephalosporins (Table S5, ESI<sup>†</sup>). This was of interest, as in preliminary work we observed some  $\beta$ -lactams react covalently (data not shown).  $\beta$ -Lactam antibiotics form stable acyl-enzyme complexes with bacterial nucleophilic serine enzymes; they inhibit other nucleophilic serine enzymes including proteases and  $\beta$ -lactamases<sup>16</sup> and nucleophilic cysteine enzymes.<sup>17</sup>

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

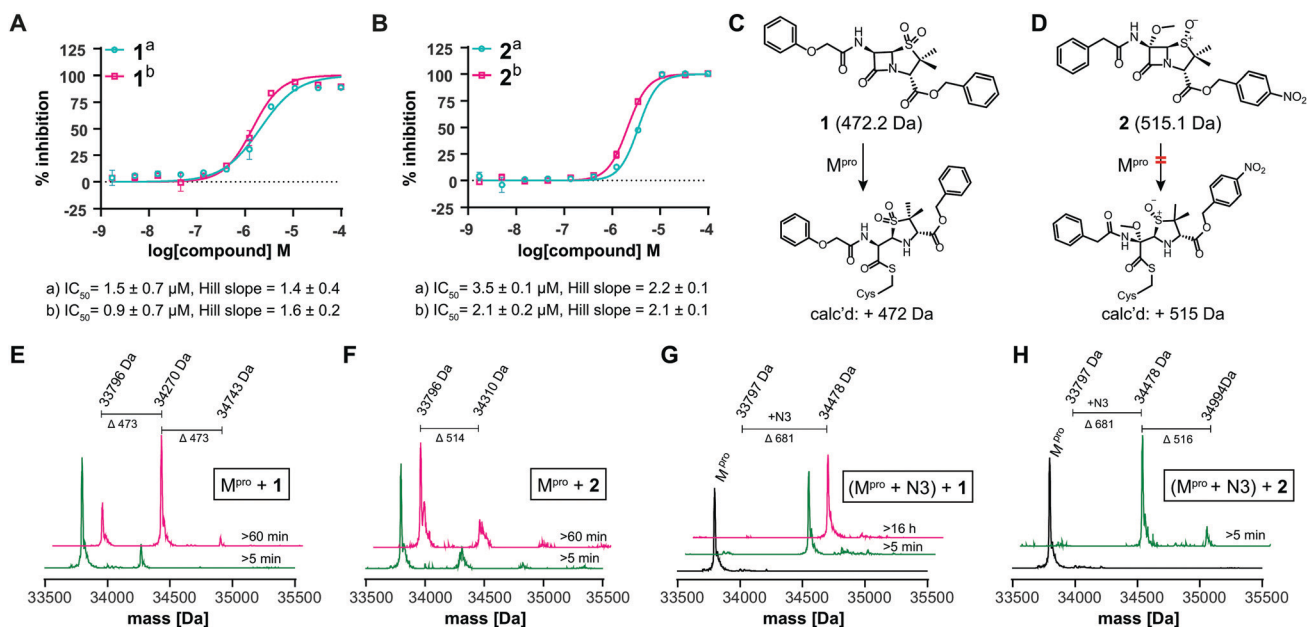




**Fig. 2** N3 dependent reaction monitoring the active site selectivity of inhibitors. (A) Reaction of N3 with the M<sup>pro</sup> active site Cys-145.<sup>1</sup> (B) N3, (C) ebselen and (D) TPCK modifies M<sup>pro</sup> in a covalent manner. (E) Ebselen covalently modifies multiple M<sup>pro</sup> cysteine residues in the presence of N3. (F) TPCK does not covalently modify M<sup>pro</sup> in the presence of N3, suggesting that it selectively reacts with active site Cys-145. Black spectra: wild-type M<sup>pro</sup> (33796 Da).

though cephalosporin C Zn(II) salt and cephalosporin C Na(I) salt inhibited. However, the IC<sub>50</sub>s for cephalosporin C Zn(II) salt and ZnCl<sub>2</sub> were similar, indicating much of the inhibition is due to Zn(II) ions (Fig. S16, ESI<sup>†</sup>), as observed for cephalosporin C Zn(II) salt inhibition of other enzymes.<sup>7b</sup>

We further investigated β-lactam reactions with M<sup>pro</sup> using a diverse set of β-lactams (Fig. S17, ESI<sup>†</sup>). Though most β-lactams were inactive (IC<sub>50</sub> > 100 μM), two penicillin esters manifested IC<sub>50</sub>s < 5 μM, *i.e.* **1**: a penicillin V sulfone C3 benzyl ester (IC<sub>50</sub> ~ 1.5 μM), and **2**: a derivative of penicillin G sulfoxide C3 p-nitrobenzyl ester (IC<sub>50</sub> ~ 1.5 μM).



**Fig. 3** β-Lactams inhibit M<sup>pro</sup>. IC<sub>50</sub>s 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. IC<sub>50</sub>s are means of two independent repeats each composed of technical duplicates (*n* = 2 ± SD). Proposed reaction of **1** (C) and **2** (D) with M<sup>pro</sup>. (E) A single molecule **1** covalently modifies M<sup>pro</sup>. (F) **2** does not efficiently modify M<sup>pro</sup> through covalent reaction. Conditions: 1 μM M<sup>pro</sup>, 20 μM β-lactam, 20 mM HEPES, pH 7.5, 50 mM NaCl. (G) **1** does not covalently modify M<sup>pro</sup> preincubated with N3, suggesting **1** reacts with Cys-145. (H) **2** does not efficiently react with M<sup>pro</sup> preincubated N3. Conditions: 1 μM M<sup>pro</sup> preincubated with 3 μM N3, 20 μM **1** or **2**, 20 mM HEPES, pH 7.5, 50 mM NaCl. Black spectra: wild-type M<sup>pro</sup> (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  $\beta$ -lactams inhibited, though more weakly (Fig. S17, ESI<sup>†</sup>). 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).<sup>1</sup> Structures of  $M^{\text{PRO}}$  complexed with a  $\beta$ -lactam were not obtained; however, docking studies reveal potential of **1** and **2** to bind favourably at the active site (Fig. S18, ESI<sup>†</sup>), 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  $\beta$ -lactam sub-families (Fig. S19–S21, ESI<sup>†</sup>). In some cases, *e.g.* clavulanate (Fig. S19H, ESI<sup>†</sup>) and moxalactam (Fig. S21F, ESI<sup>†</sup>), (partial) inhibitor fragmentation was observed. There was no clear correlation between a propensity to react covalently and  $M^{\text{PRO}}$  inhibition; in some cases evidence for partial covalent modification, but no inhibition was observed (Fig. S17, ESI<sup>†</sup>). 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  $M^{\text{PRO}}$  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  $M^{\text{PRO}}$  assays enabling analysis of inhibition by both substrate depletion/product formation. The method complements reported *in vitro*  $M^{\text{PRO}}$  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  $M^{\text{PRO}}$  modification and use of  $M^{\text{PRO}}$  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  $\beta$ -lactams can inhibit  $M^{\text{PRO}}$  non-covalently, the observation that some react with and inhibit  $M^{\text{PRO}}$  by covalent active site modification should promote interest in the development of inhibitors for  $M^{\text{PRO}}$  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  $\beta$ -lactam containing  $M^{\text{PRO}}$  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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