Mass spectrometry reveals potential of β-lactams as SARS-CoV-2 M pro inhibitors
Mass spectrometry reveals potential of β-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 β-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) is a COVID-19 treatment target. M<sup>pro</sup> along with the papain-like protease (PL pro), processes initially translated viral polyproteins to give cleaved M<sup>pro</sup> assay for identifying new inhibitors and testing known drugs.

Most reported M<sup>pro</sup> assays measure fluorescence, as precended for other protease assays. Whilst efficient, such methods do not simultaneously monitor substrate depletion/product formation and some compounds interfere with fluorescence. 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. 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. Here we report how such an assay enabled identification of new M<sup>pro</sup> inhibitors, including β-lactams, the most important antibacterial class.

We developed conditions for an SPE-MS based SARS-CoV-2 M<sup>pro</sup> assay (0.15 μM M<sup>pro</sup>, 2.0 μM TSAVLQ/SGFKR-NH₂, 20 mM HEPES, pH 7.5, 50 mM NaCl) using protein prepared as reported. Isolated M<sup>pro</sup> was found to be active when monomeric/dimeric forms of M<sup>pro</sup>, 2.0 mM TSAVLQ/SGFRK-NH₂, was cleaved to give TSAVLQ and SGFRK-NH₂ fragments (Fig. S1, ESI†). Kinetic parameters were determined for the 11-mer substrate (K<sub>m</sub> = 14.4 μM; k<sub>cat</sub> = 2.7 min<sup>−1</sup>), both by monitoring substrate cleavage and terminal product formation (Fig. S1c and D). Kinetic parameters for the 37-mer substrate were also investigated; a 2-fold increase in k<sub>cat</sub> in 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/SDFRK(Dnp)K with a fluorescence energy transfer (FRET) assay (27 000 M<sup>−1</sup> s<sup>−1</sup>, as reported and in our hands). Steady state kinetics for a 37-mer substrate were also investigated; a 2-fold increase in k<sub>cat</sub> in M<sup>pro</sup> determined by SPE-MS (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 related SARS-CoV M<sup>pro</sup> reveal similar k<sub>cat</sub> 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.

Next, the SPE-MS assay was validated for inhibition studies with ebselen, N<sub>3</sub>, disulfiram, and boceprevir using the 11-mer TSAVLQ/SDFRK-NH₂ substrate (Table 1 and Fig S3, ESI†). The ebselen IC<sub>50</sub> was ~0.09 μM under standard conditions (0.15 μM M<sup>pro</sup>, 2.0 μM TSAVLQ/SDFRK-NH₂ i.e. [S] ≪ K<sub>m</sub>, 20 mM HEPES, pH 7.5, 50 mM NaCl at ambient temperature) compared to an IC<sub>50</sub> of ~0.67 μM using a FRET assay (0.2 μM M<sup>pro</sup>, 20 μM Mca–AVLQ/SDFRK(Dnp)K i.e. [S] ≈ K<sub>m</sub>, 50 mM Tris–HCl, pH 7.3, 1 mM EDTA, 30 °C) (Table 1, entry 1; Fig. S3D, ESI†).

† Electronic supplementary information (ESI) available: Experimental details and inhibition data. See DOI: 10.1039/d0cc06870e
We optimized the assay for studying covalent modifications with a higher Mₚro concentration being used to enable robust analyses (1 µM Mₚro), though IC₅₀ 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 treated and untreated Mₚro. Following N₃ preincubation, sometimes with more than one reaction being observed (Fig. S9–S15, ESI†). Active site selectivity was investigated using N₃ treated and untreated Mₚro. Following N₃ treatment, in some cases, active sites were N₃ blocked, suggesting reaction with Cys-145 active site was N₃ blocked, suggesting reaction with Cys-145 (Fig. S3, ESI†). We exploited selective reaction of N₃ to test selectivity of other nucleophilic cysteine enzymes; they inhibit other nucleophilic cysteine enzymes. 17

Table 1 IC₅₀S of selected Mₚro inhibitors determined using SPE-MS assays compared to those obtained using FRET assays

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ (SPE-MS) [µM]</th>
<th>IC₅₀ (SPE-MS) [µM]</th>
<th>IC₅₀ (FRET) [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ebselen</td>
<td>0.09 ± 0.07</td>
<td>0.09 ± 0.07</td>
<td>0.67 ± 0.09</td>
</tr>
<tr>
<td>N₃</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>n.d.</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>0.60 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>9.35 ± 0.18</td>
</tr>
<tr>
<td>Boceprevir</td>
<td>11.0 ± 4.8</td>
<td>9.2 ± 5.5</td>
<td>2.70 ± 0.05</td>
</tr>
</tbody>
</table>

**Table 1**: IC₅₀S of selected Mₚro inhibitors determined using SPE-MS assays compared to those obtained using FRET assays.

We optimized the assay for studying covalent modifications with a higher Mₚro concentration being used to enable robust analyses (1 µM Mₚro), though IC₅₀ 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 treated and untreated Mₚro (Fig. 2A, B and Fig. S4, ESI†), consistent with structural work revealing Cys-145 reaction (Fig. 1B and Fig. S5, Table S2, ESI†). We exploited selective reaction of N₃ to test selectivity of other nucleophilic 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 µM compound (Fig. S7, ESI†). Excellent Z′-factors 13 and signal to noise ratios reveal the assay robustness (Fig. S7, ESI†). In addition to ebselen (identification of which validates the method), diverse inhibitors (≥ 80% at a fixed 20 µM 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†).

IC₅₀S were determined for compounds with ≥ 80% inhibition at 20 µM, excluding known interference compounds. 15 Auranofin (IC₅₀ ~ 1.5 µM; reported IC₅₀ ~ 0.5 µM 14), an α-chloroketone (TPCK) (IC₅₀ ~ 0.8 µM), IPA-3 (IC₅₀ ~ 0.1 µM), and 5-thioguanine (IC₅₀ ~ 13.5 µM) are some of the more potent inhibitors (Fig. S8, ESI†). Some of these covalently modified Mₚro, sometimes with more than one reaction being observed (Fig. S9–S15, ESI†). Active site selectivity was investigated using N₃ treated and untreated Mₚro. Following N₃ treatment, in some cases, e.g. TPCK and N₃-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 Mₚro were diminished when the active site was N₃ blocked, suggesting reaction with Cys-145 might alter the Mₚro conformation (Fig. S9 and S11, ESI†).

The screen identified β-lactam drugs as potential Mₚro 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-lactamases 16 and nucleophilic cysteine enzymes. 17

Studies on cephalosporins identified as potential inhibitors from the screen revealed no substantial covalent Mₚro modification,
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 Mpro using a diverse set of β-lactams (Fig. S17, ESI†). Though most β-lactams were inactive (IC50 > 100 μM), two penicillin esters manifested IC50s < 5 μM, i.e. 1: a penicillin V sulfone C3 benzyl ester (IC50 ~ 1.5 μM), and 2: a derivative of penicillin G sulfoxide C3

![Fig. 2](image1.png)

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

![Fig. 3](image2.png)

**Fig. 3** β-Lactams inhibit Mpro. 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 ± SD). Proposed reaction of 1 (C) and 2 (D) with Mpro. (E) A single molecule 1 covalently modifies Mpro through covalent reaction. Conditions: 1 μM Mpro, 20 μM β-lactam, 20 mM HEPES, pH 7.5, 50 mM NaCl. (G) 1 does not covalently modify Mpro preincubated with N3, suggesting 1 reacts with Cys-145. (H) 2 does not efficiently react with Mpro preincubated N3. Conditions: 1 μM Mpro preincubated with 3 μM N3, 20 μM 1 or 2, 20 mM HEPES, pH 7.5, 50 mM NaCl. Black spectra: wild-type Mpro (33796 Da).
p-nitrobenzyl ester (IC$_{50}$ ~ 3.5 µM), 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 Mpro 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, cepham, 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 Mpro 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 Mpro 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 Mpro assays enabling analysis of inhibition by both substrate depletion/product formation. The method complements reported in vitro Mpro 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 Mpro modification and use of Mpro 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 Mpro non-covalently, the observation that some react with and inhibit Mpro by covalent active site modification should promote interest in the development of inhibitors for Mpro 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 Mpro 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.

Notes and references