SARS-CoV-2 M^{pro} Assay for Discovery and Characterisation of Active SARS-CoV-2 Inhibitors

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1 ABSTRACT

- A promising anti-viral drug target is the Main Protease (M^{pro} or 3CL protease) of SARS-CoV-2 which is a critical component of viral replication due to its ability to cleave polyproteins pp1a and pp1ab and yield various non-structural proteins with functions such as viral mRNA methylation and host DNA unwinding. Proof of concept that modulation of M^{pro} activity protects against infection has been demonstrated by Pfizer with PAXLOVID™ (PF-07321332; ritonavir) recently entering Phase 2/3 clinical trials (1).
- At Charles River Early Discovery, we have developed a fluorogenic M^{pro} biochemical assay that detects the cleavage of a peptide substrate, derived from the N-terminal autocleavage sequence of the protease (TSAVLQ-|-SGFRK). The assay has been robustly optimised in terms of protein concentration, steady-state kinetic parameters and linearity.
- Influenced by the achievements of the COVID Moonshot project (2), and associated Diamond Light Source X-Chem fragment-screen (3), we performed a fragment-screen composed of diverse fluorinated fragments. Conventionally, ^{19}F fragments provide a benefit to NMR as the fluorine atom is not present within proteins, resulting in low background, however screening using a functional biochemical platform is possible if assay DMSO tolerance is good and the technology employed is not particularly sensitive to compound-mediated interference.
- Here we present the results of this screen that yielded thirty reproducibly active compounds with IC_{50} values down to 300 µM, some of which could offer suitable starting points for further SARS-CoV-2 antiviral drug discovery efforts. Furthermore, we describe the rapid generation of apo SARS-CoV M^{pro} crystals and introduce our internal crystal structure which will be a key and versatile tool to understand the structure activity relationship (SAR) of these active compounds. And in the longer term it will be employed to drive medicinal chemistry efforts, in addition to validating hits from any virtual screens.

2 BIOLOGICAL CHARACTERISATION

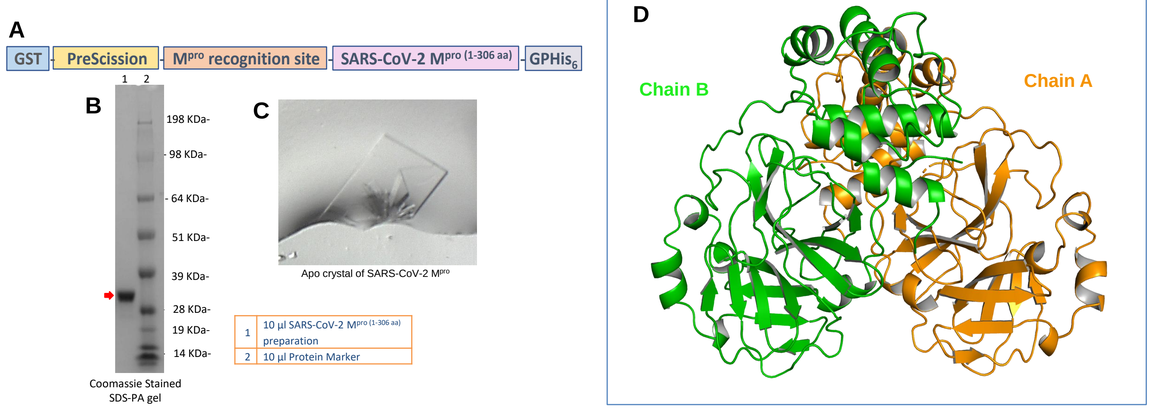


Figure 1: **SARS-CoV-2 M^{pro} expression & crystallography.** We recombinantly expressed and purified to homogeneity SARS-CoV-2 M^{pro} following the construct design reported in the literature (2). This construct design allowed the expression of SARS-CoV-2 M^{pro} with purification and expression enhancing tags (A). The tags were removed during the purification thereby leading to SARS-CoV-2 M^{pro} with native N and C termini. This was crucial for the purpose of this study since any additional amino acid at the N-terminus of SARS-CoV-2 M^{pro} would affect the activity of the target (Xue at al. 2007). SARS-CoV-2 M^{pro} was expressed as a soluble protein in BL21 (DE3) upon 1mM IPTG induction at 18C for 16 h. SARS-CoV-2 M^{pro} was purified to homogeneity in three steps by Ni-NTA affinity chromatography, His$_6$-tag cleavage upon PreScission protease treatment followed by reverse Ni-NTA affinity chromatography and then a final size exclusion chromatography (B). SARS-CoV-2 M^{pro} was crystallized in its APO form using vapor diffusion method. Crystals were obtained in 0.1 NaCl, 0.1 M Tris pH 8.0, 7% w/v PEG 20000 and diffracted to a 2.5 Å resolution SARS-CoV-2 M^{pro} at 5mg/ml hanging drops in 0.1 NaCl, 0.1 M Tris pH 8.0, 11% w/v PEG 20000 (C). (D) A cartoon representation of the SARS-CoV-2 M^{pro} structure, showing the dimerized protein obtained at 2.49Å.

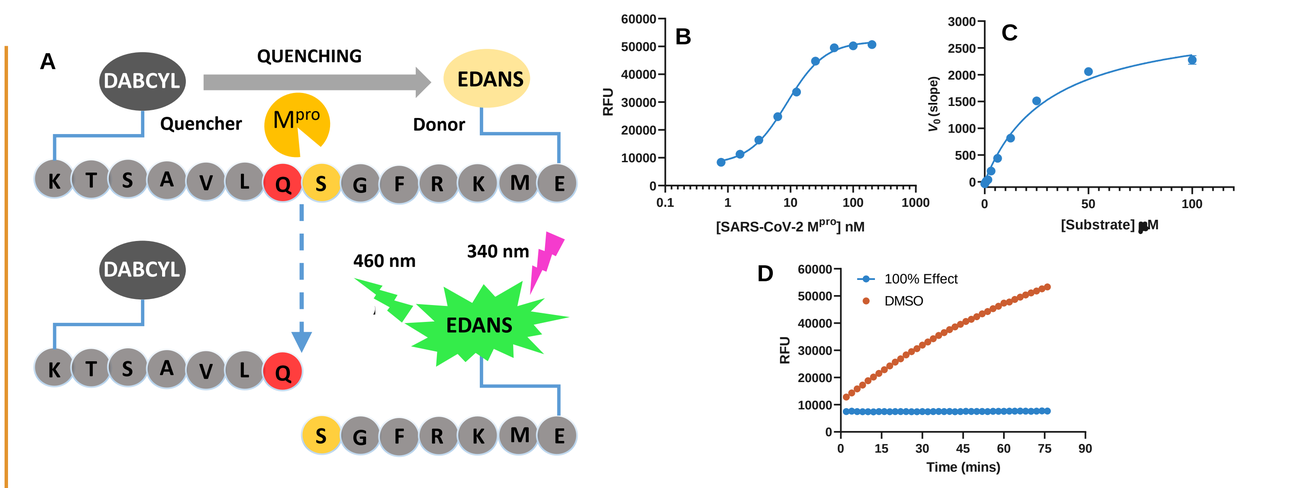
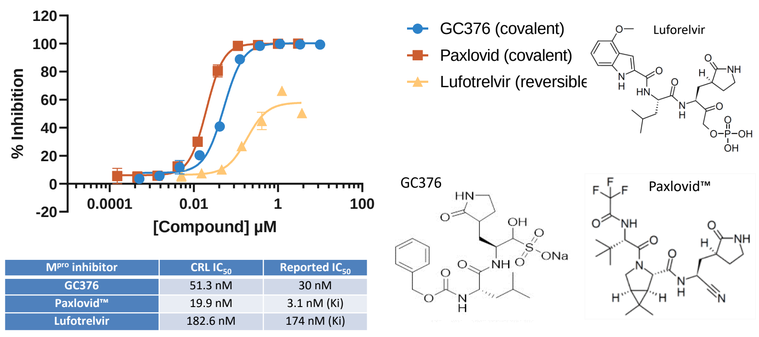


Figure 2: **SARS-CoV2-M^{pro} assay optimisation.** (A) Protease assay principle. The peptide substrate composed of the autocleavage sequence of M^{pro} is tagged with Dabcyl and Edans fluorescent moieties. In the absence of the protease, the Edans emission is suppressed by the proximity of the Dabcyl quencher; upon M^{pro}-mediated cleavage at the serine/glutamine cleavage site, Edans quenching is relieved due to the Dabcyl portion of peptide dissociation which leads to an increase in florescence at Ex. 340nm, Em. 460nm.
(B) Determination of optimal M^{pro} screening concentration. The M^{pro} concentration selected for screening was 15 nM which is within the linear range and close to the M^{pro} EC_{50} of 9 nM.
(C) Characterisation of the steady state kinetic parameters for the M^{pro} peptide substrate, Dabcyl-K TSAVLQSGFRK ME-Edans-NH2. The substrate K_m was determined to be 31 µM and a concentration of 10 µM was selected for screening.
(D) Determination of optimal incubation. At 15 nM M^{pro} and 10 µM peptide an incubation time of 30 minutes was selected for inhibitor screening and profiling where the reaction is still linear and within the initial rate of the reaction. This incubation period yields a signal:background of ~5.0 with robust Z' of 0.91.

3 ASSAY VALIDATION

GC376 is a broad-spectrum covalent antiviral compound that has been typically used as a veterinary drug for treatment of feline infectious peritonitis virus (FIPV) and has recently been shown to inhibit SARS-CoV-2 M^{pro} with an IC_{50} ~30 nM. PAXLOVID™ (PF-07321332; ritonavir) and Lufotrelvir (PF-07304814) are M^{pro} inhibitors, developed by Pfizer with PAXLOVID™ recently entering Phase 2/3 clinical trials. Inhibition constants for these compounds have been determined to be 3.1 nM and 174 nM, respectively.



M^{pro} inhibitor	CRL IC_{50}	Reported IC_{50}
GC376	51.3 nM	30 nM
Paxlovid™	19.9 nM	3.1 nM (Ki)
Lufotrelvir	182.6 nM	174 nM (Ki)

Figure 3: **M^{pro} assay validation using known M^{pro} covalent and non-covalent inhibitors.** IC_{50} determinations were carried out by assaying compounds in duplicate as 10 point 2-fold serial dilutions with a top concentration of 100, 10 or 3 µM, depending on expected potency, using the primary M^{pro} assay. Data generated after 30 minutes was normalised and used to produce inhibition curves in order to generate IC_{50} values using a 4-parameter fit model in Graphpad Prism software. All compounds showed the expected potency, however, Lufotrelvir appeared to interfere with this assay format at concentrations >3 µM, as shown by a significant hook effect that was omitted to improve fitting.

4 FLUORINATED FRAGMENT SCREEN

- ^{19}F-NMR provides a powerful tool in fragment-based drug discovery. Because the fluorine atom is not present in biological molecules ^{19}F-NMR has no background providing clear signals with high sensitivity
- The fragment team at CRL have reviewed the latest trends in fragment library design and applied them to establish a new 500 member ^{19}F labelled library primarily for screening by NMR, but in this case via a functional biochemical assay

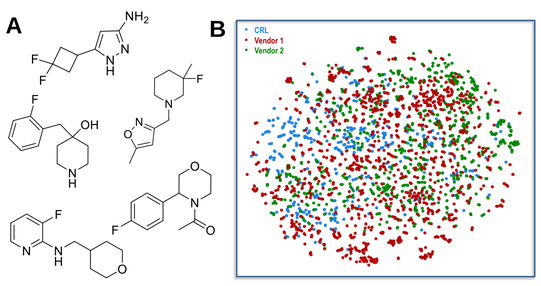


Figure 4: The new CRL ^{19}F Fragment Library contains a high level of structural diversity and compares well to other vendors covering some areas of chemical space not exemplified within the other commercial libraries. (A) Examples of CRL ^{19}F Fragment Library structures. (B) t-SNE plot representing the structurally diversity of the new CRL ^{19}F Fragment Library compared to some commercial vendor libraries.

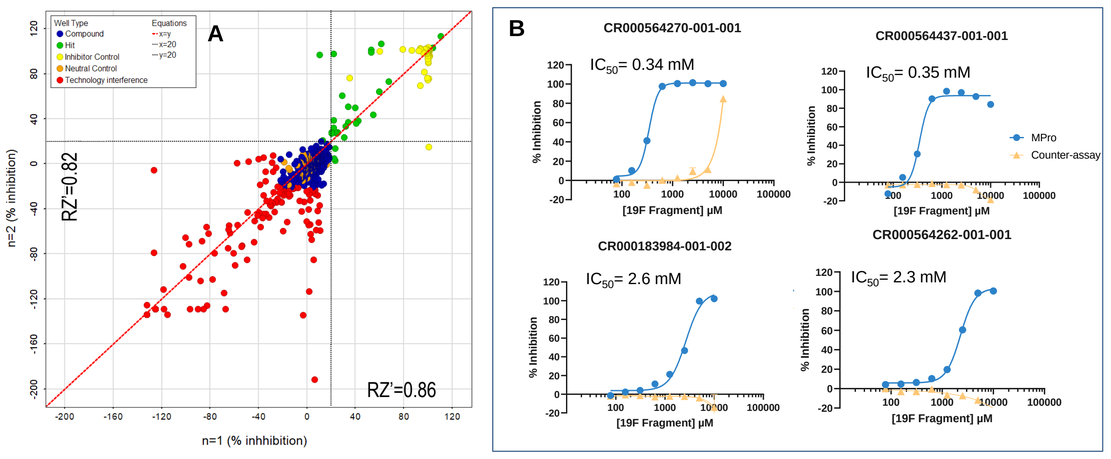


Figure 5: Correlation analysis of 500 fluorinated fragments tested at 1 mM in the M^{pro} fluorometric enzyme assay. (A) Data represented by inhibition rate were obtained from two independent experiments. Most of the test compounds overlaid tightly with the neutral controls, however ~30% of the compounds gave negative inhibition values down to almost -200% (red points) which is suggestive of compound intrinsic autofluorescence leading to compound-mediated technology interference. 30 compounds showed reproducible inhibition outside of the assay noise cut-off of 20% inhibition (green points), calculated using the mean plus 3xSD of the neutral controls.
(B) IC_{50} determinations were carried out by assaying compounds in duplicate as 8 point 2-fold serial dilutions with a top concentration of 10 mM using either the primary M^{pro} assay or a counter-assay where M^{pro} was omitted, and a fluorescent product of the reaction was employed to determine any fluorescence-quenching compounds that could appear to be false positives. All 30 single shot hits reconfirmed in IC_{50} mode with two showing reasonable potency at ~300 µM and no significant technology interference. Example curve profiles of the more potent compounds are shown.

5 SUMMARY

The CRL SARS-CoV-2 assay and apo crystal structure are resources that can be accessed by any prospective new clients who wish to embark on the COVID-19 anti-viral drug discovery journey. Coronavirus Main Protease selectivity assays can also be established rapidly within CRL to support these efforts

- We have established a robust and sensitive biochemical fluorogenic assay to measure the activity of the Main Protease (M^{pro}, 3CL) from SARS-CoV-2, and performance has been validated using well-characterised M^{pro} inhibitors, including the Pfizer clinical candidate, Paxlovid™.
- This assay was employed to screen the new Charles River Fluorinated Fragment Library which yielded thirty tractable M^{pro} inhibitors with IC_{50}s as low as 300 µM; these could provide starting points for future anti-viral drug discovery projects for any prospective new clients who wish to develop these promising hits further.
- To complement the fragment screening effort and facilitate future compound optimisation/virtual screening, we have solved the apo crystal structure of full-length M^{pro} at 2.49Å. In the near future a sub-set of the active fragments will be selected for soaking with the preformed apo M^{pro} crystals in order to obtain protein-ligand complexes to be used in driving future medicinal chemistry efforts forward.

References:
(1) An oral SARS-CoV-2 M^{pro} inhibitor clinical candidate for the treatment of COVID-19, Owen R et al., Science, 374, 6575, 2nd Nov 2021, p1586-1593
(2) COVID Moonshot: Open science discovery of SARS-CoV-2 Main Protease inhibitors by combining crowdsourcing, high-throughput experiments, computational simulations, and machine learning, Achdout, H, et al., 30th October 2020
(3) COVID MoonShot – Taking fragments to impact/Fragment screening to fight COVID-19, diamond.ac.uk