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Challenges of short substrate analogues as SARS-CoV-2 main protease inhibitors

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ABSTRACT

Specific anti-coronaviral drugs complementing available vaccines are urgently needed to fight the COVID-19 pandemic. Given its high conservation across the betacoronavirus genus and dissimilarity to human proteases, the SARS-CoV-2 main protease (M^{pro}) is an attractive drug target. SARS-CoV-2 M^{pro} inhibitors have been developed at unprecedented speed, most of them being substrate-derived peptidomimetics with cysteine-modifying warheads. In this study, M^{pro} has proven resistant towards the identification of high-affinity short substrate-derived peptides and peptidomimetics without warheads. 20 cyclic and linear substrate analogues bearing natural and unnatural residues, which were predicted by computational modelling to bind with high affinity and designed to establish structure–activity relationships, displayed no inhibitory activity at concentrations as high as 100 μM. Only a long linear peptide covering residues P_6 to P_5' displayed moderate inhibition (K_i = 57 μM). Our detailed findings will inform current and future drug discovery campaigns targeting M^{pro}.

With over 200 million reported cases and 4 million deaths,[1] the ongoing COVID-19 pandemic is among the most devastating pandemics in human history.[2] Specific antiviral drug candidates targeting SARS-CoV-2 are urgently needed to complement available vaccines and prepare for future coronavirus outbreaks.[3] Inspired by the successful discovery of HIV and HCV protease inhibitors and their development into drugs,[4] coronaviral proteases are currently among the most promising targets.[5–7]

The betacoronavirus RNA genome encodes two proteases, the papain-like protease (PL^{pro}) and the main protease (M^{pro} or $3CL^{pro}$), which process the viral polyproteins pp1a and pp1ab into smaller non-structural proteins that assemble the replisome.[8] M^{pro} is structurally conserved across SARS-CoV-1, MERS-CoV and SARS-CoV-2, which may allow the development of pan-coronaviral drugs.[7,9] The majority of polyprotein cleavage events are performed by M^{pro}, making it an attractive drug target. M^{pro} forms a homodimer and is a cysteine protease with distinct substrate specificity ranging from P_4 to P_1' (using the nomenclature of Schechter and Berger),[10] with a particularly strong preference for glutamine in P_1.[7,11] No human host proteases with similar substrate recognition are known, rendering M^{pro} an ideal drug target with respect to off-target effects.[5–7]

Before the emergence of SARS-CoV-2, M^{pro} had already attracted attention as a potential drug target against the related coronaviruses SARS-CoV-1 and MERS-CoV,[12] which emerged in 2002 and 2012, respectively. None of the small molecules and peptidomimetics reported to inhibit M^{pro} of SARS-CoV-1 and MERS-CoV were developed further into antiviral drugs.[13,14] Substrate-derived peptidomimetics relied on electrophilic reactive groups modifying the catalytic cysteine residue (commonly known as cysteine warheads) to achieve sufficient affinity. Inhibitors included Michael acceptors,[15–20] aldehydes,[20–24] aldehyde prodrugs,[25,26] α-ketoamides,[27] epoxides and aziridines,[28,29] and α-halomethyl ketones.[30,31]

Since the emergence of SARS-CoV-2 in late 2019, several more M^{pro} inhibitors have been discovered at unprecedented speed. The same dependence on reactive warheads prevails for substrate-derived peptides and peptidomimetics. Warheads employed include Michael acceptors,[32] aldehydes,[33,34] aldehyde prodrugs,[35] α-ketoamides,[36] vinylsulfones,[11] azanitriles,[37] and ketones.[38] In April 2021, Pfizer revealed the orally available M^{pro} inhibitor PF-07321332, which is a short substrate analogue featuring a C-terminal nitrile warhead.[39] Very recently, a cyclic peptide has been reported, which binds to SARS-CoV-2 M^{pro} without forming a covalent bond.[40] With an IC_{50} value of about 160 μM, however, the activity of this compound is many orders of magnitudes below those of substrate-based analogues with warheads.

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Table 1
Cyclic and linear M^{pro} substrate analogues assessed in this study.

Compound [a]	Sequence[b]	Form	Activity[c] against SARS-CoV-2 M^{pro}
1	Cys-Val-Val-Leu-Gln-Cpa-NH_2	cyclic	IC_{50} > 100 μM
2	Cys-Ala-Arg-Leu-Gln-Cpa-NH_2	cyclic	IC_{50} > 100 μM
3	Cys-Abu-Tle-Cyl-Gln-Cpa-NH_2	cyclic	IC_{50} > 100 μM
4	Cys-Thr-Thr-Cyl-Gln-Cpa-NH_2	cyclic	IC_{50} > 100 μM
5	Cys-Gln-Leu-Val-Val-Cpa-NH_2	cyclic	IC_{50} > 100 μM
6	Cys-Val-Leu-Gln-Ser-Cpa-NH_2	cyclic	IC_{50} > 100 μM
7	Cys-Ala-Ala-Val-Leu-Gln-Ala-Cpa-NH_2	cyclic	IC_{50} > 100 μM
8	Cys-Ala-Abu-Tle-Cyl-Gln-Ala-Cpa-NH_2	cyclic	IC_{50} > 100 μM
9	Cys-Abu-Tle-Cyl-Gln-Ser-Cpa-NH_2	cyclic	IC_{50} > 100 μM
10	Cys-Ala-Ala-Val-Leu-Gln-Ala-Ala-Cpa-NH_2	cyclic	IC_{50} > 100 μM
11	Cys-Ala-Ala-Val-Leu-Gln-Ser-Cpa-NH_2	cyclic	IC_{50} > 100 μM
12	Cys-Val-Val-Leu-Glu-Cpa-NH_2	cyclic	IC_{50} > 100 μM
13	Cys-Ala-Arg-Leu-Glu-Cpa-NH_2	cyclic	IC_{50} > 100 μM
14	Cys-Ala-Ala-Val-Leu-Asn-Ala-Cpa-NH_2	cyclic	IC_{50} > 100 μM
15	Cys-Phe-Gln-Ser-Lys-Cpa-NH_2	cyclic	IC_{50} > 100 μM
16	Cys-Thr-Ser-Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg-Lys-Cpa-NH_2	cyclic	IC_{50} > 100 μM
17	DCP Dab(Cys)-Abu-Tle-Leu-Gln-Lys(Cys)-NH_2	stapled/cyclic	IC_{50} > 100 μM
18	Abu-Tle-Cyl-Gln-Cpa-NH_2	linear	IC_{50} > 100 μM
19	Ac-Abu-Tle-Leu-Gln-NH_2	linear	IC_{50} > 100 μM
20	Thz-Ala-Ala-Val-Leu-Gln-Ala-Cpa-NH_2	linear	IC_{50} > 100 μM
21	Ac-Ser-Thr-Ser-Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg-Lys-Phe-NH_2	linear	IC_{50} = 71 μM K_i = 57 μM

[a] Structural formulas of **1–21** are shown in Fig. S1.
[b] List of three letter codes of unnatural amino acids and stapling reagent: Abu: L-2-aminobutanoic acid; Cpa: L-2-amino-3-(2-cyanopyridin-4-yl)-propanoic acid; Cyl: L-2-amino-3-cyclopropylpropanoic acid; Dab: L-2,4-diaminobutanoic acid; DCP: 2,6-dicyanopyridine; Thz: L-thiazolidine-4-carboxylic acid; Tle: L-2-amino-3,3-dimethylbutanoic acid.

[c] Activity determined in FRET activity assays with 25 μM substrate and 25 nM enzyme for IC_{50} determination or with 10 μM, 20 μM, 35 μM and 50 μM substrate and 12.5 nM enzyme for K_i determination.

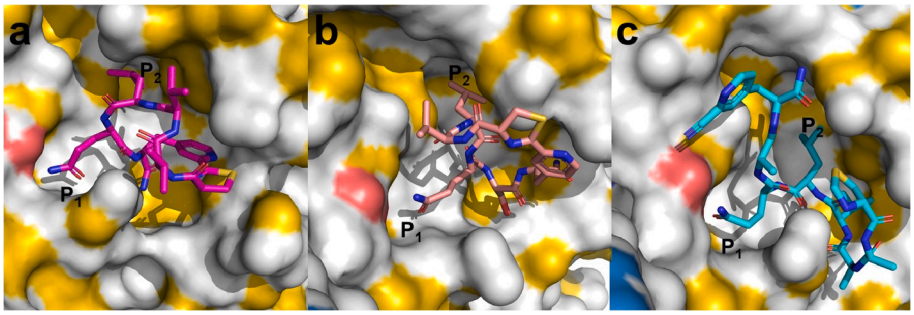

Fig. 1. Docking poses of compounds (a) **1**, (b) **6**, and (c) **20** against a dimeric X-ray crystal structure of SARS-CoV-2 M^{pro} (PDB: 6XQT). Protein shading was realized with the YRB highlighting script by Hagemans et al.[55]

Table 2
Glide GScores and binding free energies of compounds **1**, **6** and **20** docked with M^{pro} (PDB: 6XQT).

Compound	Glide GScore [kcal/mol]	Binding free energy[a] [kcal/mol]
1	−7.373	−60.47
6	−6.695	−58.85
20	−10.094	−58.50

[a] Prime/MM-GBSA calculation performed in Maestro 2019–1, Schrödinger.

Reactive warheads pose the risk of pronounced off-target effects, potentially compromising the advancement of lead compounds into clinical drugs.[41] Therefore, we set out to investigate the possibility of high-affinity short substrate-based SARS-CoV-2 M^{pro} inhibitors without warheads. We were particularly interested in exploring short cyclic substrate analogues with higher proteolytic stability than linear peptides.

Inspired by previous successes with generating nanomolar cyclic inhibitors of the Zika virus protease NS2B-NS3,[42–45] we designed various cyclic and linear analogues of the substrate amino acid sequence of SARS-CoV-2 M^{pro} (Table 1). We applied our in-house peptide-cyclization technique, which is based on the unique reactivity of 2-cyanopyridine and *N*-terminal cysteine or analogues.[42,43] The peptide sequences explored cover the entire substrate range from P_6 to P_5' (most being short peptides comprising only 4–6 residues of the substrate recognition sequence), and included unnatural amino acids where those have been reported as suitable replacement for canonical amino acids in covalent M^{pro} inhibitors (Table 1).[7,11]

Initially, we chose two short cyclic peptides (**1**, **6**) and one short linear peptide (**20**) for molecular modelling and docking experiments. All three peptides cover major recognition motifs of M^{pro}. Compound **1** features a non-prime site substrate recognition sequence (VVLQ, P_4 – P_1), while compound **6** also includes a prime site residue (VLQS, P_3 – P_1'). Peptide **20** has a P_5 – P_1' recognition sequence and serves as a linear control, as the 2-cyanopyridine cannot react with the *N*-terminal thiazolidine. Molecular docking of **1**, **6** and **20** with the dimer structure of SARS-CoV-2 M^{pro} (PDB: 6XQT) predicted promising binding orientations and interactions, especially with the S_1 and S_2 sub-cavity of the active site (Fig. 1), similar to previously co-crystallized peptidomimetics. The three compounds also showed almost similar predicted binding energies from computational docking experiments (Table 2) and were predicted to interact with critical active-site residues *via* hydrogen bonds (Fig. S3).[46]

Encouraged by the computational predictions, we designed, synthesized, and purified 21 substrate-derived peptides (Table 1). Compounds **1**–**16** were prepared using standard Fmoc solid-phase peptide synthesis (SPPS) and the aforementioned side-chain-to-tail cyclization strategy.[42] The method was successfully applied to more hydrophobic peptides and was compatible with sequences as long as 13 amino acids without major impact on the reaction yield. Compound **17** was synthesized following our in-house peptide stapling approach post Fmoc SPPS.[43] All of these chemical transformations have proven to be biocompatible and deliver high-affinity ligands of viral proteases.[42,43] Peptides **18**–**21** were designed as linear analogues for comparison.

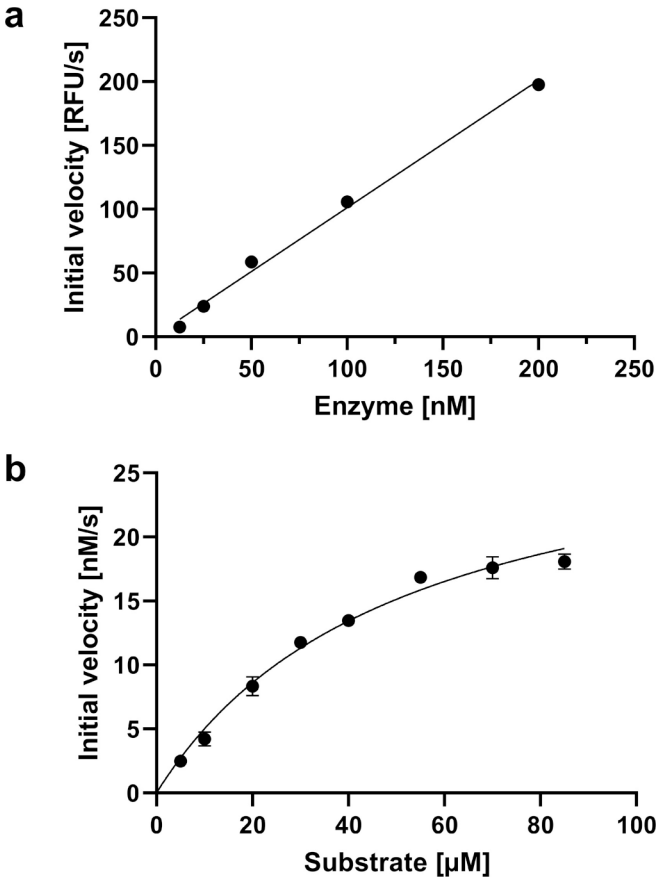

Fig. 2. Activity assay of M^{pro}. **(a)** Linear dependence of M^{pro} activity (expressed by initial velocity) from M^{pro} concentration using 25 μM FRET substrate. **(b)** Michaelis-Menten kinetics using 25 nM M^{pro}. K_M = 51 μM, k_{cat} = 1.2 s^{-1}.

To assess M^{pro} inhibition by the substrate analogues **1**–**21**, we employed an established M^{pro} inhibition assay using the FRET-based substrate DABCYL-KTSAVLQ↓SGFRKM-E(EDANS)–NH_2 and

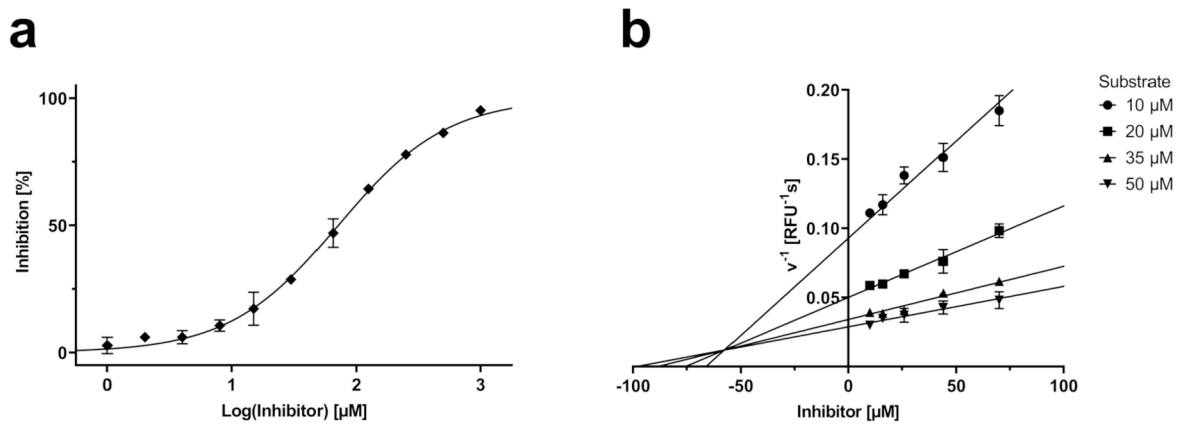

Fig. 3. (a) Dose-response curve of M^{pro} FRET assay and compound **21**. IC_{50} = 71 μM. (b) M^{pro} inhibition of compound **21** at multiple FRET substrate concentrations visualized in a Dixon plot. K_i = 57 μM.

recombinant SARS-CoV-2 M^{pro}.[21,36] M^{pro} dimer formation was confirmed by size exclusion chromatography and NMR spectroscopy (not shown). EDTA and DTT were added to the assay buffer to exclude any interferences from metal ions, oxidation, or cysteine modification. The use of reducing agent has proven to be particularly crucial to avoid nonspecific inhibition as, for example, observed for the covalent M^{pro} modifier ebselen.[47] We also confirmed that the enzymatic activity depends linearly on the M^{pro} concentration (Fig. 2**a**) and determined Michaelis Menten kinetics (Fig. 2**b**), which yielded a k_{cat}/K_M ratio (23,500 $M^{-1}s^{-1}$) that is consistent with other studies.[35,36,48]

In stark contrast to the promising computational results, compound **1** showed no inhibition of M^{pro}, even at the highest assayed concentration of 100 μM. We did not pursue tests at higher concentrations due to potential solubility problems, and because acceptable drug candidates are expected to show nanomolar or even picomolar affinities in biochemical assays.[49] In order to investigate whether the lack of inhibition at 100 μM was a compound-specific result caused by peptide length or cyclization constraints, we explored additional cyclic and linear analogues (Table 1). Compounds **2**–**4** and **17**–**19** are short analogues of **1** encompassing four natural and unnatural amino acids from P_4 to P_1. Compound **5** was designed as a *retro*-peptide analogue of compound **1**. We further expanded the substrate recognition sequence towards the non-prime site residue P_5 and prime site residues P_1' and P_2' in compounds **6**–**11**, **16** and **20**–**21**. We also tested compounds **12**–**14**, where the P_1 glutamine was replaced by glutamate or asparagine. Compound **15** was inspired by the low-affinity inhibitor reported by Kreutzer et al.[40] Remarkably, none of the peptides **1**–**20** displayed IC_{50} values below 100 μM in our well-validated M^{pro} activity assay (Table 1).

To further explore this finding, we performed NMR studies of uniformly $^{15}N/^{2}H$-labeled M^{pro} with compounds **1** and **7**. Neither compound induced any significant chemical shift perturbations in ^{15}N-HSQC spectra at 100 μM, confirming that these cyclic substrate analogues do not bind to M^{pro} at concentrations relevant to drug design (Fig. S7).

Additionally, we carried out molecular dynamics simulations (MDS) in triplicate with 900 ns total simulation time for compound **1** docked to the SARS-CoV-2 M^{pro} dimer. One of the three simulations revealed large fluctuations in the root-mean-square deviation (RMSD) between **1** and the M^{pro} dimer during a short interval between 248 ns and 256 ns (Fig. S4), corresponding to **1** diffusing out of the active site briefly before returning to occupy it until the end of the simulation (Fig. S5). In the remaining two simulations, **1** remained bound within the active site for the duration, albeit undergoing conformational fluctuations, particularly in the orientation of the Gln (P_1) and Leu (P_2) sidechains of **1**. Representative structures generated from the first cluster for all replicates revealed major differences in the positioning of these two sidechains compared to the molecular docking (Fig. S6). In contrast to the docked structure, in which the P_1 Gln of **1** interacted with the S_1 subpocket residues Leu141 and Glu166, the same sidechain does not interact to a similar degree with the S_1 subpocket in the MDS. The position of the Leu sidechain in **1** (P_2) also deviates from the pose observed in the docked structure. The results of this MDS study suggest that cyclic peptide **1** binds weakly and reversibly to the active site of M^{pro}, which may contribute to its poor *in vitro* activity.

It is clear from this study that short peptides without a warhead cannot establish high affinity interactions to the M^{pro} dimer. Our study also suggests that cyclic substrate analogues are not a suitable alternative to address the insufficient affinity of linear peptides, a strategy that has previously been successful with other viral proteases.[42–44] It is possible that our specific cyclization linkers are the cause of this observation; however, Kreutzer et al., who used an unrelated cyclization chemistry, equally failed to produce cyclic substrates with sufficient affinity.[40] It is notable that peptide **16**, which is a long (13 amino acids) cyclic analogue of the assay substrate, did not display inhibition at 100 μM. Only its linear analogue **21** showed moderate inhibition with an IC_{50} value of 71 ± 5 μM (Fig. 3**a**). Compound **21**, which is an acetylated analogue of the FRET substrate, is a competitive inhibitor with a K_i value of 57 ± 10 μM as confirmed by a Dixon plot (Fig. 3**b**). Its similarity to the natural M^{pro} substrate sequences and inhibition mode suggest that it acts as a competitive substrate. Thus, since peptide **16**, which is a cyclic version of **21**, did not show inhibition at 100 μM, it is reasonable to conclude that the macrocyclic peptide may be too constrained to bind the active site of the M^{pro} dimer in a high affinity conformation.

Our results reveal major challenges associated with the discovery of short substrate-based M^{pro} inhibitors without electrophilic warheads. Substrate analogues of shorter lengths did not show any significant activity, while a longer linear analogue displayed moderate affinity. Previously successful strategies, including cyclization and the use of unnatural amino acids, did not help to overcome these challenges. The lack of affinity of cyclic substrate analogues described here and previously by Kreutzer et al.[40] is particularly noteworthy as computational work in both studies predict binding to the active site with poses very similar to ligands observed in crystal structures. Given that our non-covalent inhibitors appear to be unusually ineffective against M^{pro}, it is not surprising that the first generation of SARS-CoV-2 M^{pro} inhibitors discovered at the beginning of the COVID-19 pandemic in 2020 were substrate derived covalent inhibitors bearing α-ketoamide, aldehyde, and Michael acceptor reactive groups.[32,33,36] It should be noted that the substrate specificity of M^{pro} may be overruled by the electrophilicity of a warhead, as previously demonstrated.[21] It should also be noted that the first generation of drugs targeting the HCV protease NS3-4A, such as telaprevir and boceprevir, required covalent warheads (α-ketoamides) as well,[50,51] while subsequent generations of drug candidates, such as faldaprevir or danoprevir, no longer require warheads.[52,53] It is thus not inconceivable that substrate-inspired inhibitors of M^{pro} without

warheads may eventually become available, although present inhibitors still require warheads to boost affinity. Perhaps the larger diversity of peptide libraries available from phage or mRNA displays might help to overcome these challenges.[54]

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128333.

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