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A head-to-head comparison of the inhibitory activities of 15 peptidomimetic SARS-CoV-2 3CLpro inhibitors

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ABSTRACT

The COVID-19 pandemic caused by SARS-CoV-2 has created an unprecedented global health emergency. As of July 2021, only three antiviral therapies have been approved by the FDA for treating infected patients, highlighting the urgent need for more antiviral drugs. The SARS-CoV-2 3C-like protease (3CLpro) is deemed an attractive drug target due to its essential role in viral polyprotein processing and pathogenesis. Indeed, a number of peptidomimetic 3CLpro inhibitors armed with electrophilic warheads have been reported by various research groups that can potentially be developed for treating COVID-19. However, it is currently impossible to compare their relative potencies due to the different assays employed. To solve this, we conducted a head-to-head comparison of fifteen reported peptidomimetic inhibitors in a standard FRET-based SARS-CoV-2 3CLpro inhibition assay to compare and identify potent inhibitors for development. Inhibitor design and the suitability of various warheads are also discussed.

In December 2019, a spate of pneumonia clusters were reported by a number of healthcare facilities in Wuhan, Hubei province, China, with symptoms such as a sore throat, dry cough, fever, headache, fatigue and breathing.\(^1,2\) Genome sequencing revealed that the disease was caused by a coronavirus with 80% nucleotide sequence identity to the severe acute respiratory syndrome coronavirus (SARS-CoV), the pathogen responsible for the 2002–2004 pandemic which originated from Foshan, Guangdong province, China.\(^3,7\) This novel coronavirus was named ‘severe acute respiratory syndrome coronavirus 2’ (SARS-CoV-2) by the International Committee on Taxonomy of Viruses\(^8\) and the disease was named ‘coronavirus disease 2019’ (COVID-19) by the World Health Organization (WHO) in 2020. SARS-CoV-2 has since spread globally, infecting more than 182 million people worldwide and killing more than 3.9 million as of 3 July 2021.\(^9\) Although a number of vaccines have been granted authorization, only three antiviral therapies (Remdesivir, Casirivimab + Imdevimab and Sotrovimab) have so far been approved by the United States Food and Drug Administration (FDA) for treating infected patients.\(^10-12\) On 20 November 2020, the WHO recommended against the use of Remdesivir for hospitalized SARS-CoV-2 patients citing the lack of evidence that it improves survival and other outcomes,\(^13\) highlighting the urgent need for more effective antiviral drugs.

The SARS-CoV-2 3C-like protease (3CLpro; also known as main protease or Mpro) is deemed an attractive drug target due to its involvement in cleaving the viral polyprotein at a minimum 11 sites to form essential viral proteins required for virus replication and pathogenesis.\(^14-17\) In addition, it has no reported human homologues, hence reducing the risk of off-target side-effects by 3CLpro inhibitors.\(^17\) This cysteine protease recognizes and binds Leu-Gln, Phe-Gln and Val-Gln peptide sequences and cleaves the peptide bond at the C-terminus of Gln.\(^16-18\) Hence, such peptides designed with C-terminal electrophilic warheads have been shown to inhibit CoV 3CLpro (Fig. 1). Examples of electrophilic warheads include aldehydes,\(^19,21\) ethyl propenoate,\(^28\) hydroxymethylketone,\(^22\) hydroxymethyl sulfonic acid,\(^16,23\) ketomides,\(^16,24,25\) and ketobenzothiazoles.\(^26,27\) This peptide-warhead strategy has led to the approval of Boceprevir and Telaprevir in 2011 (Fig. 1), antiviral drugs that target the hepatitis C virus (HCV) NS3 protease and used for treating HCV infections,\(^28\) suggesting the same strategy can be applied for SARS-CoV-2 antiviral drugs.

Although no peptidomimetic inhibitors have so far been approved for treating SARS-CoV-2 infections, they have been reported by various research groups (see Table 1 and references cited therein). Intriguingly, HCV NS3 protease inhibitors Boceprevir and Telaprevir were also reported to inhibit SARS-CoV-2 3CLpro, suggesting that they can be repurposed for treating COVID-19.\(^16,25,29\)
Unfortunately, it is currently impossible to compare their relative inhibitory potencies due to the different experimental protocols and protease constructs used by the various research groups. To solve this, we conducted a head-to-head comparison of fifteen reported coronavirus 3CLpro peptidomimetic inhibitors using a fluorescence resonance energy transfer (FRET)-based CoV-2 3CLpro inhibition assay. IC_{50} values were then compared to identify potent inhibitors that have the potential for further development as antiviral drugs. In addition, inhibitor design and the suitability of various electrophilic warheads will also be discussed.

Test compounds 2i, 3, 11a, 11b, 17, 25c, GC-373 and TG-0205221 were synthesized based on reported procedures cited in Table 1 and their spectral data are found in the supplemental file. Boceprevir was purchased from Selleckchem (USA). Calpeptin, MG-115 and MG-132 were purchased from Santa Cruz Biotechnology (USA). GC-376 and Telaprevir were purchased from BOC Sciences (China). PF-0835231 was custom-synthesized by WuXi AppTec (China).

SARS-CoV-2 3CLpro expression and purification is based on a published procedure\textsuperscript{16} and our modified protocol is found in the supplemental file. A highly sensitive FRET-based protease assay was developed to identify inhibitors of 3CL proteases based on a published protocol.\textsuperscript{30} The peptide substrate (Dabcyl)KTSAVLQSGFRKM(Glu) (EDANS) was synthesized by Genscript (USA). The test compounds were

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**Fig. 1.** Reported peptidomimetic coronavirus 3CLpro inhibitors.
3-fold serially diluted in 100% DMSO to 15 concentrations, starting at values, e.g. (1) SARS-CoV-2 3CLpro inhibitory activities (µM) of various reported inhibitors. Numbers in parentheses represent relative potencies based on in-house IC_{50} values, e.g. (1) = most potent.

| Compound | In-house IC_{50} (µM) | Literature IC_{50} or K_{i} (µM) | CoV-1 or CoV-2 3CLpro | Literature reference |
|----------|----------------------|-----------------------------------|------------------------|----------------------|
| 2i       | (8) 0.094 ± 0.006     | 1.7 (IC_{50})                     | CoV-1                  | 26                   |
| 3        | (9) 0.286 ± 0.014     | 0.66 (K_{i})                      | CoV-1                  | 19                   |
| 11a      | (3) 0.014 ± 0.001     | 0.053 ± 0.005 (IC_{50})           | CoV-2                  | 20                   |
|          |                      | 0.031 ± 0.003 (IC_{50})           | CoV-2                  | 23                   |
| 11b      | (4) 0.023 ± 0.003     | 0.040 ± 0.002 (IC_{50})           | CoV-2                  | 20                   |
| 17       | (7) 0.065 ± 0.007     | 0.007 (IC_{50})                   | CoV-1                  | 24                   |
| 25c      | >10                   | 21.0 (IC_{50})                    | CoV-1                  | 27                   |
| Boceprevir | >10                | 4.13 ± 0.61 (IC_{50})             | CoV-2                  | 16                   |
|           |                      | 5.40 ± 1.53 (IC_{50})             | CoV-2                  | 29                   |
| Calpeptin| >10                   | 10.69 ± 0.28 (IC_{50})            | CoV-2                  | 16                   |
| GC-376   | (6) 0.042 ± 0.001     | 0.40 ± 0.05 (IC_{50})             | CoV-2                  | 21                   |
| GC-376   | (5) 0.034 ± 0.001     | 0.030 ± 0.008 (IC_{50})           | CoV-2                  | 16                   |
| MG-115   | >10                   | 2.14 ± 0.97 (IC_{50})             | CoV-2                  | 16                   |
| MG-132   | >10                   | 3.90 ± 1.01 (IC_{50})             | CoV-2                  | 16                   |
| PF-0835231| (1) 0.008 ± 0.001    | 0.004 ± 0.0003 (IC_{50})          | CoV-1                  | 22                   |
| Telaprevir| >10               | 11.47 (IC_{50})                   | CoV-2                  | 25                   |
| TG-0205221| (2) 0.009 ± 0.001   | 0.053 (K_{i})                     | CoV-1                  | 19                   |

Type of protease used in the assay as reported in the literature.

3-fold serially diluted in 100% DMSO to 15 concentrations, starting at 3.33 mM. 1.5 µl of the serially diluted compounds were transferred to a black 384 well assay plate (Cat. 781900, Greiner). 23.5 µl of 2.13X concentration of SARS-CoV-2 Chis-3CLpro enzyme prepared in assay buffer was added to the compounds and incubated for 30 mins at 25 °C. 25 µl of 2X concentration of peptide substrate was added to the assay plate and incubated at 37 °C for 1.5 h. The final assay contained 12.5 mM of enzyme, 6 µM substrate and 3% DMSO in assay buffer containing 50 mM HEPES at pH 7.5, 100 mM NaCl, and 0.01% Triton X-100 and 1 mM DTT. The starting test compound concentration started at 100 µM. The FRET signal was measured using an excitation wavelength of 340 nm (UV[TRF] 340/60 nm, Barcode 101), emission wavelength of 490 nm (DSPPsiOn 486/10 filter, Barcode 220) and Lance/DELFIA D400 single mirror (Barcode 412) on Envision plate reader (2104 EnVision Multi-label Plate Readers, Perkin Elmer). The dose–response curves were fitted with a variable slope using GraphPad Prism software (GraphPad, USA) to determine a compound’s IC_{50}. Experiments were conducted in duplicates and IC_{50} were determined from two independent experiments.

The IC_{50} of the test compounds, along with their reported inhibitory data and literature references, are summarised in Table 1.

The next two most potent inhibitors are peptide aldehydes 11a and 11b, exhibiting IC_{50}s of 14 and 23 nM respectively (Table 1). Like PF-0835231, both contain a 2-carboxyindole moiety whose NH and CO were shown to be H-bonded to 3CLpro’s Glu166 backbone CO and NH using X-ray crystallography (PDB codes 6LZE and 6M0K). However, like TG-0205221, we believe the aldehyde moieties will pose pharmacological liabilities stated vide supra. One possible solution is to substitute the aldehyde with a hydroxymethylketone warhead seen in PF-0835231 before they can be reconsidered for drug development.

The fifth and sixth most potent compounds, GC-376 and GC-373, are peptides with a hydroxymethyl sulfonic acid and aldehyde warhead respectively (IC_{50}s 34 and 42 nM respectively; Table 1). GC-376’s hydroxymethyl sulfonic acid is a prodrug moiety that transforms into an aldehyde warhead in physiological conditions to react and form a covalent bond with 3CLpro’s active site Cys145. However, we believe this will still result in metabolic liabilities for GC-376 once the reactive aldehyde is formed in the bloodstream as discussed vide supra. Similarly, we predict the aldehyde warhead of GC-373 will pose metabolic problems as stated earlier.

GSK’s peptide ketoamide 17 is the seventh most potent inhibitor in

![Fig. 2. Two-dimensional depiction of PF-07304814 (blue) covalently bound to Cys145 (red) in the SARS-CoV-2 3CLpro active site based on co-crystal structure 6XHM.pdb. The covalent bond formed between Cys145 and PF-07304814 is depicted as a bold red line. Hash lines represent hydrogen bonds.](image-url)
our test panel with an IC₅₀ of 65 nM (Table 1). Designed with a ketomide warhead, we believe it possesses a high potential for further drug development as ketomides have been shown to be effective warheads in the approved oral HCV NS3 protease inhibitors Boceprevir and Telaprevir. Based on this, we believe 17 can potentially be developed into an oral anti-CoV-2 drug. Oral drugs are favoured over intravenously-administered ones as they do not cause pain during administration, do not require trained personnel to administer and thus allowing administration in home settings. These factors serve to significantly improve patient dosing compliance and we recommend it for further development to treat COVID-19.

Compound 2i with a ketobenzothiazole warhead is the eighth most potent inhibitor with an IC₅₀ of 94 nM (Table 1), suggesting that it has the potential for further drug development. Peptide ketobenzothiazoles were first reported as Rhinovirus 3C protease inhibitors by Agouron Pharmaceuticals in 2000. To our best knowledge, no peptide-ketobenzothiazole inhibitors have entered the clinic so far. A report that the benzothiazole N and S are both prone to metabolic oxidation suggests that it may become a metabolic liability although there are at least ten non-peptide drugs containing the benzothiazole moiety available in the market. Hence, we believe compound 2i deserves further investigation with careful scrutiny on its pharmacokinetic and pharmacodynamic profile.

Compound 3 (Fig. 1) is the ninth most potent inhibitor and is the sole compound with an ethyl propenoate warhead in our test panel. Peptides with this particular warhead were used as Rhinovirus 3C protease inhibitors by Agouron Pharmaceuticals in 1998. Their most advanced compound, Rupintrivir, reached phase 2 clinical trials in 1999. Although Rupintrivir was reported to be inactive against SARS-CoV-1 and SARS-CoV-2 3CLpro, its analogue compound 3 was reported to bind SARS-CoV-1 3CLpro with a Kᵢ of 660 nM although its IC₅₀ was not reported. In our SARS-CoV-2 3CLpro inhibition assay, compound 3 exhibited an IC₅₀ of 286 nM (Table 1). In our view, it would be premature to consider it for drug development due to its moderate inhibitory potency. More structure-activity relationship studies would have to be conducted to enhance its potency before reconsideration.

The remaining compounds: Boceprevir, Calpeptin, MG-115, MG-132 and Telaprevir were found to be weak inhibitors with IC₅₀ above 10 μM (Table 1), making them unsuitable for further development. It is noteworthy that they lack the P1 cyclic glutamine analogue found in most of our test compounds, suggesting that this is a critical moiety for SARS-CoV-2 3CLpro recognition. Indeed, X-ray co-crystal structures of PF-07304814, 11a and 11b bound to SARS-CoV-2 3CLpro (PDB codes 6XHM, 6L2E, 6MOK) revealed that the P1 lactam’s NH and CO are involved in H-bonding to the 3CLpro’s Phe140 backbone CO and His163 side-chain imidazole respectively (Fig. 2). This suggests that when designing new peptidomimetic 3CLpro inhibitors, an amide moiety must be present in the P1 residue.

In conclusion, a head-to-head IC₅₀ comparison of fifteen published coronavirus 3CLpro peptidomimetic inhibitors was conducted using a standard SARS-CoV-2 3CLpro inhibition assay. The most potent compound was identified to be PF-07304814 (IC₅₀ 8 nM), with a hydroxymethylketone warhead and recently entered COVID-19 clinical trials as a phospho drug. Peptide aldehydes, well-known for being highly potent protease inhibitors, populated the second to sixth spots in term of potencies (IC₅₀ 9–42 nM). However, as aldehyde warheads can potentially pose toxicity and metabolic issues, we suggest that they should first be converted to hydroxymethylketone or ketoamide warheads before being re-evaluated. GS’x peptide ketoamide 17 was the seventh most potent inhibitor (IC₅₀ 65 nM) and we believe it has the potential to be further developed as an oral antiviral drug based on the successes of Boceprevir and Telaprevir. Peptides with ketobenzothiazole and ethyl propenoate warheads (compounds 2i and 3) were relatively less potent (IC₅₀ 94 and 286 nM respectively) and we opine they should not be used in their current forms. Lastly, we note that test compounds without a P1 amide moiety exhibited IC₅₀ of >10 μM, making them unsuitable for further drug development. This strongly suggests that a P1 amide moiety is critical to the design of future SARS-CoV-2 3CLpro peptidomimetic inhibitors.

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.

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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.2021.128263.

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