

# A warhead substitution study on the coronavirus main protease inhibitor Nirmatrelvir.

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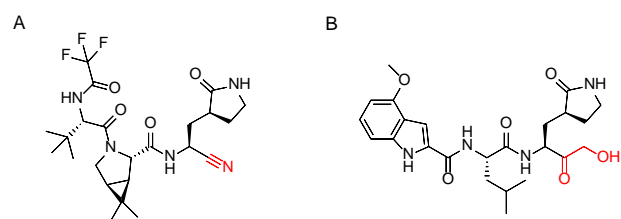
**ABSTRACT:** The SARS-CoV-2 pandemic is currently causing an unprecedented global health emergency since its emergence in December 2019. In December 2021, the FDA granted emergency use authorization to nirmatrelvir, a SARS-CoV-2 main protease inhibitor for treating infected patients. This peptidomimetic is designed with a nitrile warhead which forms a covalent bond to the viral protease. Herein, we investigate nirmatrelvir analogs with different warheads and their inhibitory activities. In addition, antiviral activities against human alphacoronavirus 229E was also investigated along with a cell-based assay. We discovered that the hydroxymethylketone and ketobenzothiazole warheads were equipotent to the nitrile warhead, suggesting that these analogs can also be used for treating coronavirus infections.

**KEYWORDS:** SARS-CoV-2, COVID-19, 3CL<sup>pro</sup>, Mpro, nirmatrelvir, Paxlovid

The current severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic is causing an unprecedented global health emergency. Infection symptoms include sore throat, dry cough, pyrexia, lethargy, body aches, headaches, anosmia and ageusia. More serious symptoms include pneumonia and dyspnea which can lead to death. First reported in Wuhan, China in December 2019, it was named ‘coronavirus disease 2019’ (COVID-19) by the World Health Organization (WHO) in 2020.<sup>1-3</sup> It has since spread worldwide and by 10 July 2022, more than 550 million cases and 6 million fatalities have been reported,<sup>4</sup> highlighting the urgent need for antiviral drugs.

On 22 December 2021, the US Food and Drug Administration (FDA) granted Pfizer’s nirmatrelvir (Figure 1A) Emergency Use Authorization (EUA) for treating infected patients.<sup>5-8</sup> The drug inhibits the SARS-CoV-2 main protease, also known as 3C-like protease (3CL<sup>pro</sup>), that plays a crucial role in cleaving the coronavirus polyprotein to form smaller essential proteins required for virus replication and pathogenesis.<sup>6,9-11</sup> Indeed, 3CL<sup>pro</sup> is deemed an attractive drug target as it has no known human homologues and hence, inhibitor drugs should lack off-target side-effects.<sup>6,9</sup> Nirmatrelvir is a peptidomimetic resembling 3CL<sup>pro</sup>’s natural polypeptide substrate leucine-glutamine recognition sequence.<sup>6</sup> Its P1 residue is a 5-membered lactam resembling glutamine while its P2 residue is a rigidified leucine analog (Figure 1A). Upon binding to 3CL<sup>pro</sup>’s active site, its C-terminal nitrile reacts with the Cys145 –SH moiety to form a covalent thioimidate bond, thereby inhibiting 3CL<sup>pro</sup>.<sup>6</sup>

We were surprised by the choice of the nitrile warhead found on nirmatrelvir as a past report revealed peptidomimetics with nitrile warheads possessed only moderate 3CL<sup>pro</sup> inhibitory activities (IC<sub>50</sub>s 5 to 49 μM).<sup>12</sup> In addition, peptide nitriles were also found to be inactive towards enterovirus 3C proteases.<sup>13</sup> Intrigued, we conducted a structure-activity relationship study on 10 nirmatrelvir analogs with various warheads to investigate how different warheads affected inhibitory activities. In addition, their inhibitory activities against human coronavirus (HCoV) 229E 3CL<sup>pro</sup> was also investigated with a cell-based assay to gauge their potential to be used as pan-coronavirus inhibitors for future coronavirus pandemics.



**Figure 1.** (A) Nirmatrelvir. (B) PF-00835231. Warheads depicted in red.

Nirmatrelvir and the test compounds were synthesized based on reported methods and procedures can be found in the Supporting Information. The biochemical 3CL<sup>pro</sup> inhibition assay is based on a published procedure<sup>11</sup> and details can be found in our recent paper.<sup>14</sup> The 3CL<sup>pro</sup> peptide substrate

(DABCYL)-KTSAVLQSGFRKM-(Glu)(EDANS) was synthesized by Genscript. The cell-based HCoV 229E inhibition assay protocol can be found in the Supporting Information.

Nirmatrelvir and its 10 analogs with various electrophilic warheads were synthesized and their inhibitory activities against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> were determined using a FRET-based biochemical assay, collated in Table 1. HCoV 229E was first reported in Europe in 1966 and is now globally distributed.<sup>15,16</sup> It causes a range of respiratory symptoms with varying severity, from mild upper respiratory tract irritation to potentially life-threatening bronchiolitis and pneumonia.<sup>16</sup> As its 3CL<sup>pro</sup> shares only 40% sequence identity to SARS-CoV-2 based on a BLAST search,<sup>17</sup> a 3CL<sup>pro</sup> inhibitor with inhibitory activities against both viral proteases can potentially be used as a pan-coronavirus antiviral for future coronavirus pandemics.

Our biochemical 3CL<sup>pro</sup> inhibition assay data revealed nirmatrelvir to be highly potent against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> with IC<sub>50</sub>s of 0.031 and 0.145  $\mu$ M respectively (Table 1). In addition, the cell-based HCoV 229E inhibition assay using MRC5 human lung fibroblast cells exhibited an EC<sub>50</sub> of 0.212  $\mu$ M, 3-fold less potent than the reported 0.075  $\mu$ M against SARS-CoV-2 using human bronchial epithelial cells.<sup>6</sup> These sub-micromolar EC<sub>50</sub>s suggest that nirmatrelvir can potentially be utilized as a pan-coronavirus antiviral drug for future coronavirus pandemics.

Our first test candidate (**1**) is a nirmatrelvir analog without the nitrile warhead. ‘Warheadless’ peptidomimetic inhibitors have been shown to inhibit the West Nile virus and Murray Valley encephalitis virus proteases with single-digit micromolar IC<sub>50</sub>s,<sup>18,19</sup> suggesting that a reactive electrophilic warhead may not be needed for protease inhibition. However, our biochemical 3CL<sup>pro</sup> inhibition assay data revealed **1** to be devoid of any inhibitory activities against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> (IC<sub>50</sub>s >100  $\mu$ M; Table 1), suggesting that an electrophilic warhead is essential for inhibiting coronavirus 3CL<sup>pro</sup>.

Our second candidate (**2**) replaces nirmatrelvir’s nitrile with an aldehyde. Aldehyde peptides are well-known protease inhibitors and have been reported extensively in the literature.<sup>20,21</sup> In the context of SARS-CoV-2 3CL<sup>pro</sup> inhibition, reported IC<sub>50</sub>s range from 9 nM to 11  $\mu$ M, depending on the residues used in the peptide aldehyde inhibitor.<sup>11,14,22,23</sup> Expectedly, our biochemical 3CL<sup>pro</sup> inhibition assay data revealed **2** to potentially inhibit SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> with IC<sub>50</sub>s of 0.010 and 0.016  $\mu$ M respectively (Table 1). It is noteworthy that **2** exhibited a 9-fold inhibitory activity improvement for HCoV 229E 3CL<sup>pro</sup> compared to nirmatrelvir (0.016 vs. 0.145  $\mu$ M respectively), suggesting that **2** may be more efficacious than nirmatrelvir. However, our cell-based HCoV 229E inhibition assay revealed that its EC<sub>50</sub> to be 0.770  $\mu$ M, almost 4 times less potent than nirmatrelvir (EC<sub>50</sub> 0.212  $\mu$ M), suggesting that the aldehyde moiety hindered cell penetration. In addition, aldehydes are also known to be metabolically unstable due to their susceptibility to oxidation and reduction by human liver enzymes, making them unsuitable for drug development.<sup>24,25</sup> Hence, we believe peptide aldehydes will be hampered by pharmacological liabilities and opine that peptide aldehyde **2** lacks potential for further drug development.

The third candidate (**3**) replaces nirmatrelvir’s nitrile with a primary alcohol. A peptide alcohol has been reported by Pfizer with mild inhibitory activity (IC<sub>50</sub> 68  $\mu$ M) against SARS-CoV-1 3CL<sup>pro</sup> in their 2005 patent application.<sup>26</sup> Interestingly, our biochemical 3CL<sup>pro</sup> inhibition assay data revealed **3** to be devoid of any inhibitory activity against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> (IC<sub>50</sub>s >100  $\mu$ M; Table 1). This indicates that for a warhead to react with 3CL<sup>pro</sup>’s active site Cys145, the warhead’s electrophilic carbon may need to be linear or planar (sp or sp<sup>2</sup> hybridized) and not tetrahedral (sp<sup>3</sup> hybridized).

Our fourth candidate (**4**) replaces nirmatrelvir’s nitrile with a primary amide. Peptide amides have been reported to inhibit the dengue and zika virus non-structural proteases with single- to double-digit micromolar potencies.<sup>27,28</sup> However, our biochemical 3CL<sup>pro</sup> inhibition assays revealed **4** to be impotent against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> (IC<sub>50</sub>s >100  $\mu$ M; Table 1), suggesting that primary amides, although planar, are unsuitable warheads for inhibiting coronavirus 3CL<sup>pro</sup>. The same observation was also reported in a recent publication involving a linear tetrapeptide amide with a P1 glutamine and P2 leucine against SARS-CoV-2 3CL<sup>pro</sup>.<sup>29</sup>

The fifth candidate (**5**) replaces nirmatrelvir’s nitrile with a hydroxymethylketone moiety. Peptidomimetics with this warhead have been reported to be highly potent against SARS-CoV-1 3CL<sup>pro</sup> with single- to double-digit nanomolar IC<sub>50</sub>s.<sup>30</sup> As the SARS-CoV-1 3CL<sup>pro</sup> share 96% sequence identity to SARS-CoV-2,<sup>31,32</sup> it was thus unsurprising to observe **5** exhibiting IC<sub>50</sub>s of 0.008 and 0.013  $\mu$ M against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> respectively (Table 1). It is noteworthy that these are 4- to 11-fold improvements over nirmatrelvir (IC<sub>50</sub>s 0.031 and 0.145  $\mu$ M respectively; Table 1), suggesting that a hydroxymethylketone warhead is more reactive than a nitrile. Similarly, 6 to 8 nM IC<sub>50</sub>s were reported recently for Pfizer’s indole dipeptide hydroxymethylketone PF-00835231 (Figure 1B) against SARS-CoV-2.<sup>14,33</sup> Despite this, our cell-based HCoV 229E inhibition assay revealed inhibitor **5** and nirmatrelvir to exhibit very similar EC<sub>50</sub>s (0.193 vs. 0.212  $\mu$ M; Table 1), suggesting that the hydroxymethylketone moiety was less efficient than a nitrile for cell membrane penetration. Despite this, the phosphate prodrug of PF-00835231 entered clinical trials in September 2020 for treating hospitalised COVID-19 patients (clinical trial identifier: NCT04535167).<sup>34</sup> Hence, we opine inhibitor **5** has the potential for further development as a pan-coronavirus antiviral drug.

Our sixth candidate (**6**) replaces nirmatrelvir’s nitrile with a methyl ester. A peptide methyl ester has been shown to moderately inhibit the hepatitis C virus non-structural protease by Boehringer Ingelheim researchers (IC<sub>50</sub> 17  $\mu$ M).<sup>35</sup> However, our biochemical 3CL<sup>pro</sup> inhibition assays revealed **6** to be impotent against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> (IC<sub>50</sub>s >100  $\mu$ M; Table 1), suggesting that methyl esters are unsuitable warheads for inhibiting coronavirus 3CL<sup>pro</sup>.

The seventh candidate (**7**) replaces nirmatrelvir’s nitrile with an ethylpropenoate moiety. Peptidomimetics with this warhead were first reported by Agouron Pharmaceuticals to inhibit human rhinovirus 3C protease in 1998 and were later shown to inhibit the enterovirus 3C protease with single-digit micromolar IC<sub>50</sub>s.<sup>36-39</sup> Our recent investigation showed that a 3-residue ethylpropenoate peptidomimetic designed by TaiGen Biotechnology was able to inhibit SARS-CoV-2 3CL<sup>pro</sup> with an IC<sub>50</sub> of 286 nM.<sup>14,40</sup> Interestingly, when the

warhead was incorporated into nirmatrelvir, peptidomimetic **7** exhibited comparable  $IC_{50}$ s against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> compared to nirmatrelvir (0.044 vs. 0.031  $\mu$ M and 0.290 vs. 0.145  $\mu$ M respectively; Table 1), initially highlighting **7**'s drug development potential. However, our cell-based HCoV 229E inhibition assay revealed that its  $EC_{50}$  to be 1.294  $\mu$ M, 6-fold less potent than nirmatrelvir ( $EC_{50}$  0.212  $\mu$ M), suggesting that the ethylpropenoate moiety hindered cell penetration. It is also noteworthy that rupintrivir (formerly AG7088), another peptidomimetic armed with an ethylpropenoate warhead, was abandoned by Pfizer after phase 2 clinical trials due to a lack of efficacy against rhinovirus infection.<sup>41</sup> Hence, we opine inhibitor **7** lacks drug development potential.

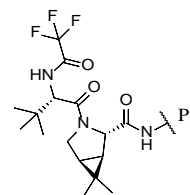
Our eighth candidate (**8**) replaces nirmatrelvir's nitrile with a ketoamide warhead. Peptide ketoamides have been approved for the treatment of hepatitis C virus infection, targeting the hepatitis C virus non-structural protease and is exemplified by the approved antivirals boceprevir and telaprevir.<sup>42</sup> Peptide ketoamides have also been reported to inhibit SARS-CoV-2 3CL<sup>pro</sup> with  $IC_{50}$ s ranging from 65 nM to 5.7  $\mu$ M, depending on the residues in the peptide ketoamide.<sup>11,14,32</sup> In our biochemical 3CL<sup>pro</sup> inhibition assays, inhibitor **8** exhibited  $IC_{50}$ s of 0.019 and 0.070  $\mu$ M against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> respectively, approximately 2-fold more potent than nirmatrelvir (Table 1). However, our cell-based HCoV 229E inhibition assay revealed its  $EC_{50}$  to be 2.365  $\mu$ M, 11-fold less potent than nirmatrelvir ( $EC_{50}$  0.212  $\mu$ M; Table 1), indicating that the ketoamide moiety hindered cell penetration. Hence, we believe peptide ketoamide **8** lacks further drug development potential.

The ninth candidate (**9**) replaces nirmatrelvir's nitrile with a benzyloxymethylketone warhead. Such peptidomimetics were first reported by SmithKline Beecham Pharmaceuticals as potent sub-micromolar cathepsin K cysteine protease inhibitors in 1999.<sup>43</sup> In our biochemical 3CL<sup>pro</sup> inhibition assays, **9** exhibited  $IC_{50}$ s of 0.027 and 0.467  $\mu$ M against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> respectively, on par with nirmatrelvir for SARS-CoV-2 3CL<sup>pro</sup> ( $IC_{50}$  0.031  $\mu$ M) but 3-fold less potent against HCoV 229E 3CL<sup>pro</sup> ( $IC_{50}$  0.145  $\mu$ M). A plausible reason could be due to steric effect caused by the warhead's benzyl moiety, hindering inhibitor **9** from binding to the HCoV 229E 3CL<sup>pro</sup> active site. Cell-based HCoV 229E inhibition assay revealed its  $EC_{50}$  to be 0.513  $\mu$ M, approximately 2-fold less potent than nirmatrelvir ( $EC_{50}$  0.212  $\mu$ M), suggesting peptidomimetic **9** to be an inferior drug candidate compared to nirmatrelvir.

Our last inhibitor (**10**) replaces nirmatrelvir's nitrile with a ketobenzothiazole warhead and was first published by Pfizer in 2021.<sup>6</sup> Such peptidomimetics were first reported in 2000 by Agouron Pharmaceuticals which could bind to rhinovirus 3C protease with nanomolar binding affinities.<sup>44</sup> Binding affinities towards SARS-CoV-2 3CL<sup>pro</sup> were recently reported to be between 8 to 230 nM<sup>6,45,46</sup> while  $IC_{50}$  ranged between 94 nM to >10  $\mu$ M, depending on the residues used in the inhibitors.<sup>14</sup> In our biochemical 3CL<sup>pro</sup> inhibition assays, **10** exhibited  $IC_{50}$ s of 0.027 and 0.239  $\mu$ M against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> respectively, similar to nirmatrelvir ( $IC_{50}$  0.031 and 0.145  $\mu$ M respectively; Table 1). Cell-based HCoV 229E inhibition assay revealed its  $EC_{50}$  to be on par with nirmatrelvir ( $EC_{50}$ s 0.212 vs. 0.242  $\mu$ M respectively; Table 1), indicating

inhibitor **10** to be a plausible candidate for further development as a pan-coronavirus antiviral for future pandemics.

**Table 1.** Inhibitory activities of nirmatrelvir and its analogs against SARS-CoV-2 and HCoV 229E arranged based on increasing molecular weights. Warheads are depicted in red. Asterisks represent potentially reactive electrophilic carbons.



| Compound     | PI | 3CL <sup>pro</sup> $IC_{50}$ ( $\mu$ M) |                   | $EC_{50}$ ( $\mu$ M) |
|--------------|----|---|-------------------|----------------------|
|              |    | SARS-CoV-2                              | HCoV 229E         | HCoV 229E            |
| Nirmatrelvir |    | 0.031 $\pm$ 0.001                       | 0.145 $\pm$ 0.017 | 0.212 $\pm$ 0.064    |
| <b>1</b>     |    | >100                                    | >100              | not tested           |
| <b>2</b>     |    | 0.010 $\pm$ 0.001                       | 0.016 $\pm$ 0.001 | 0.770 $\pm$ 0.213    |
| <b>3</b>     |    | >100                                    | >100              | not tested           |
| <b>4</b>     |    | >100                                    | >100              | not tested           |
| <b>5</b>     |    | 0.008 $\pm$ 0.001                       | 0.013 $\pm$ 0.001 | 0.193 $\pm$ 0.118    |
| <b>6</b>     |    | >100                                    | >100              | not tested           |
| <b>7</b>     |    | 0.044 $\pm$ 0.002                       | 0.290 $\pm$ 0.018 | 1.294 $\pm$ 0.664    |

|           |  |               |               |               |
|-----------|--|---------------|---------------|---------------|
| <b>8</b>  |  | 0.019 ± 0.001 | 0.070 ± 0.003 | 2.365 ± 0.792 |
| <b>9</b>  |  | 0.027 ± 0.001 | 0.467 ± 0.054 | 0.513 ± 0.095 |
| <b>10</b> |  | 0.027 ± 0.001 | 0.239 ± 0.012 | 0.242 ± 0.132 |

In conclusion, 10 nirmatrelvir analogs with varying war-heads were synthesized and their coronavirus 3CL<sup>pro</sup> inhibitory activities determined (Table 1) to gauge their potential for antiviral drug development as pan-coronavirus inhibitors. Inhibitors **2** (aldehyde), **5** (hydroxymethylketone), **8** (ketoamide) and **10** (ketobenzothiazole) were found to be more or equipotent to nirmatrelvir against SARS-CoV-2 and HCoV 229E 3CL<sup>pro</sup> based on biochemical inhibition (IC<sub>50</sub>) assays. A subsequent HCoV 229E cell-based inhibition (EC<sub>50</sub>) assay revealed inhibitors **5** and **10** to be equipotent to nirmatrelvir, indicating both are plausible candidates for further drug development as pan-coronavirus inhibitors for future coronavirus pandemics. We recommend subjecting these candidates for further biochemical IC<sub>50</sub> studies using 3CL proteases from other coronaviruses followed by animal coronavirus infection models.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthetic procedures, NMR characterization, HRMS spectra, protease production, biochemical and cell-based assay protocols (MS Word)

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

3CL<sup>pro</sup>, 3C-like protease; BLAST, basic local alignment search tool; CoV, coronavirus; COVID-19, coronavirus disease 2019; DMSO, dimethyl sulfoxide; EC<sub>50</sub>, half-maximal effective concentration; EMEM, Earles's minimum essential medium; EUA, emergency use authorization; FBS, fetal bovine serum; FDA, Food and Drug Administration; FRET, fluorescence resonance energy transfer; HCoV, human coronavirus; HCV, hepatitis C virus; HPLC, high performance liquid chromatography; IC<sub>50</sub>, half-maximal inhibitory concentration; MOI, multiplicity of infection; Mpro, main protease; SARS, severe acute respiratory syndrome; WHO, World Health Organization

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