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Letter

A Warhead Substitution Study on the Coronavirus Main Protease Inhibitor Nirmatrelvir

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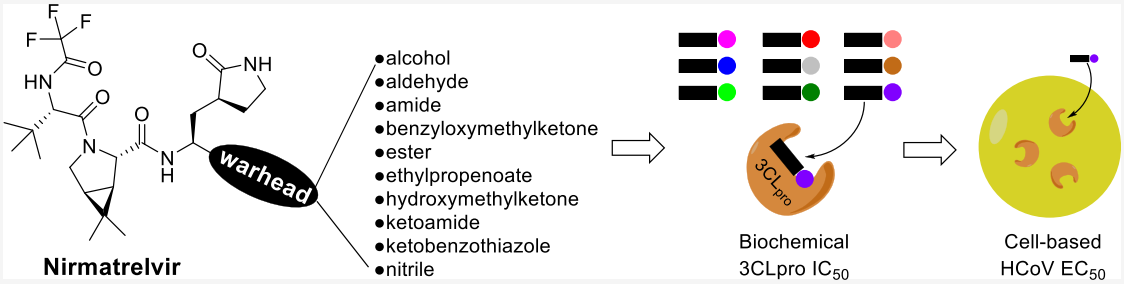

ABSTRACT: The SARS-CoV-2 pandemic is currently causing an unprecedented global health emergency since its emergence in December 2019. In December 2021, the FDA granted emergency use authorization to nirmatrelvir, a SARS-CoV-2 main protease inhibitor, for treating infected patients. This peptidomimetic 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, 3CLpro, 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.[1−3] It has since spread worldwide, and by 10 July 2022, more than 550 million cases and 6 million fatalities have been reported,[4] 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.[5−8] The drug inhibits the SARS-CoV-2 main protease, also known as 3C-like protease (3CL^{pro}), which plays a crucial role in cleaving the coronavirus polyprotein to form smaller essential proteins required for virus replication and pathogenesis.[6,9−11] Indeed, 3CL^{pro} is deemed an attractive drug target as it has no known human homologues and hence inhibitor drugs should lack off-target side effects.[6,9] Nirmatrelvir is a peptidomimetic resembling 3CL^{pro}'s natural polypeptide substrate leucine−glutamine recognition sequence.[6] 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^{pro}'s active site, its C-

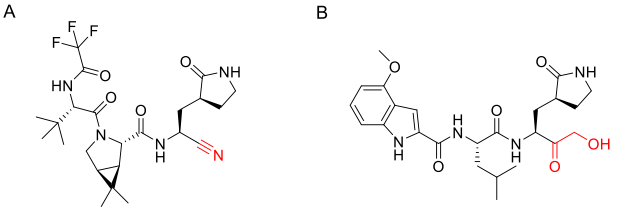

Figure 1. (A) Nirmatrelvir. (B) PF-00835231. Warheads depicted in red.

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terminal nitrile reacts with the Cys145−SH moiety to form a covalent thioimidate bond, thereby inhibiting 3CL^{pro}.[6]

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^{pro} inhibitory activities (IC_{50} values of 5−49 μM).[12] In addition, peptide nitriles were also found to be inactive toward enterovirus 3C proteases.[13] 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^{pro} was also investigated with a cell-based assay to gauge their potential to be used as pan-coronavirus inhibitors for future coronavirus pandemics.

Nirmatrelvir and the test compounds were synthesized based on reported methods and procedures can be found in the Supporting Information. The biochemical 3CL^{pro} inhibition assay is based on a published procedure[11] and details can be found in our recent paper.[14] The 3CL^{pro} 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.

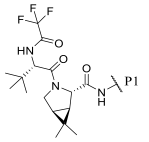
Nirmatrelvir and its 10 analogs with various electrophilic warheads were synthesized, and their inhibitory activities against SARS-CoV-2 and HCoV 229E 3CL^{pro} were determined using a FRET-based biochemical assay, collated in Table 1. HCoV 229E was first reported in Europe in 1966 and is now globally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

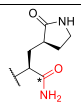
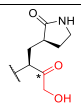
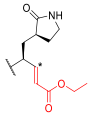
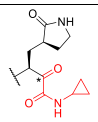
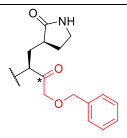
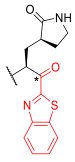
Our biochemical 3CL^{pro} inhibition assay data revealed nirmatrelvir to be highly potent against SARS-CoV-2 and HCoV 229E 3CL^{pro} with IC_{50} values of 0.031 and 0.145 μM, respectively (Table 1). In addition, the cell-based HCoV 229E inhibition assay using MRC5 human lung fibroblast cells revealed an EC_{50} of 0.212 μM, 3-fold less potent than the reported 0.075 μM against SARS-CoV-2 using human bronchial epithelial cells.[6] These sub-micromolar EC_{50} values 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_{50} values,[18,19] suggesting that a reactive electrophilic warhead may not be needed for protease inhibition. However, our biochemical 3CL^{pro} inhibition assay data revealed **1** to be devoid of any inhibitory activity against SARS-CoV-2 and HCoV 229E 3CL^{pro} (IC_{50} > 100 μM; Table 1), suggesting that an electrophilic warhead is essential for inhibiting coronavirus 3CL^{pro}.

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.[20,21] In the context of SARS-CoV-2 3CL^{pro} inhibition, reported IC_{50} values range from 9 nM to 11 μM, depending on the residues used in the peptide aldehyde inhibitor.[11,14,22,23]

Table 1. Inhibitory Activity of Nirmatrelvir and Its Analogs against SARS-CoV-2 and HCoV 229E Arranged Based on Increasing Molecular Weights[a]



		3CL^{pro} IC_{50} (μM)		EC_{50} (μM)
Compound	P1	SARS-CoV-2	HCoV 229E	HCoV 229E
Nirmatrelvir		0.031 ± 0.001	0.145 ± 0.017	0.212 ± 0.064
1		>100	>100	not tested
2		0.010 ± 0.001	0.016 ± 0.001	0.770 ± 0.213
3		>100	>100	not tested
4		>100	>100	not tested
5		0.008 ± 0.001	0.013 ± 0.001	0.193 ± 0.118
6		>100	>100	not tested
7		0.044 ± 0.002	0.290 ± 0.018	1.294 ± 0.664
8		0.019 ± 0.001	0.070 ± 0.003	2.365 ± 0.792
9		0.027 ± 0.001	0.467 ± 0.054	0.513 ± 0.095
10		0.027 ± 0.001	0.239 ± 0.012	0.242 ± 0.132

[a]Warheads are depicted in red. Asterisks represent potentially reactive electrophilic carbons.

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Expectedly, our biochemical 3CL^{pro} inhibition assay data revealed **2** to potently inhibit SARS-CoV-2 and HCoV 229E 3CL^{pro} with IC_{50} values of 0.010 and 0.016 μM, respectively (Table 1). It is noteworthy that **2** exhibited a 9-fold inhibitory activity improvement for HCoV 229E 3CL^{pro} compared to nirmatrelvir (0.016 vs 0.145 μM, respectively), suggesting that **2** may be more efficacious than nirmatrelvir. However, our cell-based HCoV 229E inhibition assay revealed its EC_{50} to be 0.770 μM, almost 4 times less potent than nirmatrelvir (EC_{50} 0.212 μM), 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.[24,25] 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_{50} = 68 μM) against SARS-CoV-1 3CL^{pro} in their 2005 patent application.[26] Interestingly, our biochemical 3CL^{pro} inhibition assay data revealed **3** to be devoid of any inhibitory activity against SARS-CoV-2 and HCoV 229E 3CL^{pro} (IC_{50} > 100 μM; Table 1). This indicates that for a warhead to react with 3CL^{pro}'s active site Cys145, the warhead's electrophilic carbon may need to be linear or planar (sp or sp^2 hybridized) and not tetrahedral (sp^3 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 nonstructural proteases with single- to double-digit micromolar potencies.[27,28] However, our biochemical 3CL^{pro} inhibition assays revealed **4** to be impotent against SARS-CoV-2 and HCoV 229E 3CL^{pro} (IC_{50} > 100 μM; Table 1), suggesting that primary amides, although planar, are unsuitable warheads for inhibiting coronavirus 3CL^{pro}. 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^{pro}.[29]

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^{pro} with single- to double-digit nanomolar IC_{50} values.[30] As the SARS-CoV-1 3CL^{pro} share 96% sequence identity to SARS-CoV-2,[31,32] it was thus unsurprising to observe **5** exhibiting IC_{50} values of 0.008 and 0.013 μM against SARS-CoV-2 and HCoV 229E 3CL^{pro}, respectively (Table 1). It is noteworthy that these are 4−11-fold improvements over nirmatrelvir (IC_{50} of 0.031 and 0.145 μM respectively; Table 1), suggesting that a hydroxymethylketone warhead is more reactive than a nitrile. Similarly, 6−8 nM IC_{50} values were reported recently for Pfizer's indole dipeptide hydroxymethylketone, PF-00835231 (Figure 1B), against SARS-CoV-2.[14,33] Despite this, our cell-based HCoV 229E inhibition assay revealed inhibitor **5** and nirmatrelvir to exhibit very similar EC_{50} values (0.193 vs 0.212 μM; 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 hospitalized COVID-19 patients (clinical trial identifier NCT04535167).[34] Hence, we opine that 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 nonstructural protease by Boehringer lngelheim researchers (IC_{50} = 17 μM).[35] However, our biochemical 3CL^{pro} inhibition assays revealed **6** to be impotent against SARS-CoV-2 and HCoV 229E 3CL^{pro} (IC_{50} > 100 μM; Table 1), suggesting that methyl esters are unsuitable warheads for inhibiting coronavirus 3CL^{pro}.

The seventh candidate (**7**) replaces nirmatrelvir's nitrile with an ethyl propenoate 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_{50} values.[36−39] Our recent investigation showed that a 3-residue ethyl propenoate peptidomimetic designed by TaiGen Biotechnology was able to inhibit SARS-CoV-2 3CL^{pro} with an IC_{50} of 286 nM.[14,40] Interestingly, when the warhead was incorporated into nirmatrelvir, peptidomimetic **7** exhibited comparable IC_{50} values against SARS-CoV-2 and HCoV 229E 3CL^{pro} compared to nirmatrelvir (0.044 vs 0.031 μM and 0.290 vs 0.145 μM respectively; Table 1), initially highlighting **7**'s drug development potential. However, our cell-based HCoV 229E inhibition assay revealed its EC_{50} to be 1.294 μM, 6-fold less potent than nirmatrelvir (EC_{50} 0.212 μM), suggesting that the ethyl propenoate moiety hindered cell penetration. It is also noteworthy that rupintrivir (formerly AG7088), another peptidomimetic armed with an ethyl propenoate warhead, was abandoned by Pfizer after phase 2 clinical trials due to a lack of efficacy against rhinovirus infection.[41] Hence, we opine that 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 nonstructural protease, and are exemplified by the approved antivirals boceprevir and telaprevir.[42] Peptide ketoamides have also been reported to inhibit SARS-CoV-2 3CL^{pro} with IC_{50} values ranging from 65 nM to 5.7 μM, depending on the residues in the peptide ketoamide.[11,14,32] In our biochemical 3CL^{pro} inhibition assays, inhibitor **8** exhibited IC_{50} values of 0.019 and 0.070 μM against SARS-CoV-2 and HCoV 229E 3CL^{pro}, 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 μM, 11-fold less potent than nirmatrelvir (EC_{50} 0.212 μM; 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.[43] In our biochemical 3CL^{pro} inhibition assays, **9** exhibited IC_{50} values of 0.027 and 0.467 μM against SARS-CoV-2 and HCoV 229E 3CL^{pro} respectively, on par with nirmatrelvir for SARS-CoV-2 3CL^{pro} (IC_{50} 0.031 μM) but 3-fold less potent against HCoV 229E 3CL^{pro} (IC_{50} 0.145 μM). 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^{pro} active site. The cell-based HCoV 229E inhibition assay revealed its EC_{50} to be 0.513 μM, approximately 2-fold less potent than nirmatrelvir (EC_{50} = 0.212 μM), suggesting peptidomimetic **9** to be an inferior drug candidate compared to nirmatrelvir.

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Our last inhibitor (**10**) replaces nirmatrelvir's nitrile with a ketobenzothiazole warhead and was first published by Pfizer in 2021.[6] Such peptidomimetics were first reported in 2000 by Agouron Pharmaceuticals and could bind to rhinovirus 3C protease with nanomolar binding affinities.[44] Binding affinities toward SARS-CoV-2 $3CL^{pro}$ were recently reported to be between 8 and 230 nM,[6,45,46] while IC_{50} values ranged between 94 nM and >10 μM, depending on the residues used in the inhibitors.[14] In our biochemical $3CL^{pro}$ inhibition assays, **10** exhibited IC_{50} values of 0.027 and 0.239 μM against SARS-CoV-2 and HCoV 229E $3CL^{pro}$, respectively, similar to nirmatrelvir (IC_{50} of 0.031 and 0.145 μM respectively; Table 1). The cell-based HCoV 229E inhibition assay revealed its EC_{50} value to be on par with that of nirmatrelvir (EC_{50} 0.212 vs 0.242 μM, respectively; Table 1), indicating inhibitor **10** to be a plausible candidate for further development as a pan-coronavirus antiviral for future pandemics.

In conclusion, 10 nirmatrelvir analogs with varying warheads were synthesized and their coronavirus $3CL^{pro}$ inhibitory activities were 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 $3CL^{pro}$ based on biochemical inhibition (IC_{50}) assays. A subsequent HCoV 229E cell-based inhibition (EC_{50}) 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 to further biochemical IC_{50} 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 at https://pubs.acs.org/doi/10.1021/acsmedchemlett.2c00260.

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

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

$3CL^{pro}$, 3C-like protease; BLAST, basic local alignment search tool; CoV, coronavirus; COVID-19, coronavirus disease 2019; DMSO, dimethyl sulfoxide; EC_{50}, 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_{50}, half-maximal inhibitory concentration; MOI, multiplicity of infection; Mpro, main protease; SARS, severe acute respiratory syndrome; WHO, World Health Organization

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