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Main protease mutants of SARS-CoV-2 variants remain susceptible to nirmatrelvir

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ABSTRACT

The COVID-19 pandemic continues to be a public health threat. Multiple mutations in the spike protein of emerging variants of SARS-CoV-2 appear to impact on the effectiveness of available vaccines. Specific antiviral agents are keenly anticipated but their efficacy may also be compromised in emerging variants. One of the most attractive coronaviral drug targets is the main protease (M^{pro}). A promising M^{pro} inhibitor of clinical relevance is the peptidomimetic nirmatrelvir (PF-07321332). We expressed M^{pro} of six SARS-CoV-2 lineages (C.37 Lambda, B.1.1.318, B.1.2, B.1.351 Beta, B.1.1.529 Omicron, P.2 Zeta), each of which carries a strongly prevalent missense mutation (G15S, T21I, L89F, K90R, P132H, L205V). Enzyme kinetics reveal that these M^{pro} variants are catalytically competent to a similar degree as the wildtype. We show that nirmatrelvir has similar potency against the variants as the wildtype. Our *in vitro* data suggest that the efficacy of the specific M^{pro} inhibitor nirmatrelvir is not compromised in current COVID-19 variants.

Since its emergence in late 2019,[1] COVID-19 has significantly impacted on societies worldwide.[2] Over 5.7 million deaths have been attributed to COVID-19, with the number of confirmed SARS-CoV-2 infections surpassing 400 million.[3] The outbreak of SARS-CoV-2 prompted multiple successful vaccine development campaigns.[4] Currently approved vaccines, such as viral vector or mRNA vaccines, successfully limited the pandemic's impact on global health.[5,6] Most COVID-19 vaccines function by stimulating an immune response against the SARS-CoV-2 spike protein (S)[7–9] but, as the spike gene has gathered considerable genetic variability,[10,11] it is a concern if the effectiveness of existing vaccines is affected by variants of SARS-CoV-2.[5,6,10,12] At the time of writing, the World Health Organization (WHO) lists five variants of concern (VOC; Alpha, Beta, Gamma, Delta, Omicron) and two variants of interest (VOI; Lambda, Mu).[13] A possible reformulation of the vaccines adjusted to currently circulating lineages of SARS-CoV-2 is being investigated.[14–16] The deployment of vaccines clearly remains the best public health measure to control the spread of SARS-CoV-2 and the severe health effects of COVID-19.[17,18]

Complementary to preventive vaccines, antiviral drugs are urgently needed to combat COVID-19.[19] Since the discovery of SARS-CoV-1 in 2003,[20] several coronaviral drug targets have been identified,[21] including the RNA-dependent RNA polymerase (RdRp, nsp12),[22] the helicase (nsp13),[23] the papain-like protease (PL^{pro}, part of nsp3)[24] and the main protease (M^{pro}, $3CL^{pro}$, nsp5).[25] Despite this, treatment options for COVID-19 are limited. Recombinant neutralizing monoclonal antibodies (mAbs) are employed in the clinical management of COVID-19, but resistance of the SARS-CoV-2 Omicron variant is a major concern.[26] The orally active drugs molnupiravir (MK-4482, EIDD-2801, Lagevrio™) and nirmatrelvir (PF-07321332, Paxlovid™ as combination drug with ritonavir as booster) were first approved for emergency use in the United Kingdom and the United States in late 2021. Molnupiravir targets RdRp by acting as a nucleoside analogue prodrug, but was originally developed against different RNA viruses.[27] Nirmatrelvir is an orally available peptidomimetic targeting M^{pro}, employing a nitrile warhead to covalently bind the catalytic cysteine residue in the active site of the protease (Fig. 1).[28]

SARS-CoV-2 M^{pro} is a homodimeric cysteine protease, which processes the majority of the viral polyproteins pp1a and pp1ab encoded by the ORF1a/b gene.[25,29] Inhibition of M^{pro} thus ultimately hinders the assembly of the replication and transcription complexes (RTCs).[25,30] The protease has a distinct recognition motif, with – in the Schechter-Berger notation[31] – preference for leucine in P_2 and especially strong preference for glutamine in P_1.[25,32] Human host proteases have different substrate specificities and it is therefore anticipated that selective inhibitors have limited off-target effects.[25]

Previous research on SARS-CoV-1 M^{pro} (which is 96% identical in

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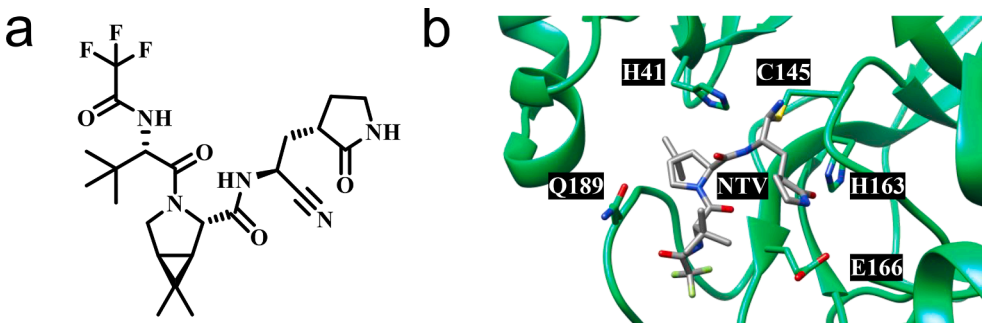
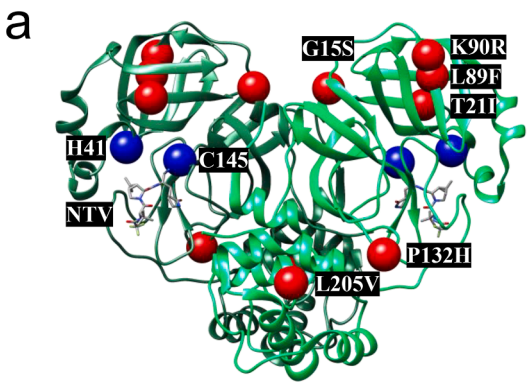


Fig. 1. SARS-CoV-2 M^{pro} inhibitor nirmatrelvir (PF-07321332).[28] (a) Chemical structure of nirmatrelvir. (b) X-ray co-crystal structure of SARS-CoV-2 M^{pro} in complex with nirmatrelvir (NTV) indicating the catalytic dyad and key interacting residues as proposed by Owen et al. (PDB: 7RFW).[28]



b

Mutation	Lineage
WT	i.a. B.1.617.2 (Delta)
G15S	C.37 (Lambda)
T21I	B.1.1.318
L89F	B.1.2
K90R	B.1.351 (Beta)
P132H	B.1.1.529 (Omicron)
L205V	P.2 (Zeta)

Fig. 2. Comparison of M^{pro} mutants. (a) X-ray co-crystal structure of SARS-CoV-2 M^{pro} in complex with nirmatrelvir (NTV) (PDB: 7RFW).[28] The sites of mutations (red) and the catalytic dyad (blue) in the two protomers (green) are indicated. (b) List of prevalent M^{pro} mutations and their corresponding SARS-CoV-2 lineage.

amino acid sequence to SARS-CoV-2 M^{pro})[25] demonstrated that missense point mutations can influence protease activity. Mutants have been identified with slightly enhanced (S284, T285, I286)[33–34] and slightly or severely reduced catalytic activity (G11, N28, S139, F140, E166, N214, R298).[33,35–39] Specifically the R298A mutation has become a tool to study the protease in its monomeric form, since it inactivates the protease by disrupting the M^{pro} dimer.[36] The present study assesses the M^{pro} mutants of emerging SARS-CoV-2 lineages. We analyzed the most widespread amino acid substitutions in SARS-CoV-2 M^{pro}, characterized them by enzyme kinetics and assessed their susceptibility to inhibition by nirmatrelvir.

Utilizing the Outbreak.info database by Scripps Research,[40] which partially operates with data provided by the GISAID Initiative,[41] we performed an analysis of the genomes of SARS-CoV-2 lineages, including the VOC and VOI. The WIV04 sequence (EPI_ISL_402124)[42] acted as wildtype (WT) reference genome. Lineage comparison[43] of VOCs and VOIs revealed three missense mutations in the M^{pro} section of the ORF1a/b gene with > 20% frequency of occurrence. The mutations are G15S, which is > 85% prevalent[44] in the Lambda VOI (or C.37, using PANGO nomenclature)[45], K90R, which is > 95% prevalent[46] in the Beta VOC (B.1.351) and P132H, which is > 95% prevalent[47] in the Omicron VOC (B.1.1.529). The Delta VOC (B.1.617.2), which was the dominant lineage for most of the second half of 2021,[48,49] did not display any particularly prevalent (>20%)[43] missense mutations within the M^{pro} part of ORF1a/b, implying that its M^{pro} is identical to that of the WT. Furthermore, we chose to investigate three additional abundant M^{pro} mutations to cover a larger variety of lineages: T21I, which is > 90% prevalent[50] in B.1.1.318, a WHO variant under monitoring (VUM),[13] L89F, which is > 95% prevalent[51] in the B.1.2 lineage, and L205V, which is > 95% prevalent[52] in the former VOI Zeta (P.2) (Fig. 2b).

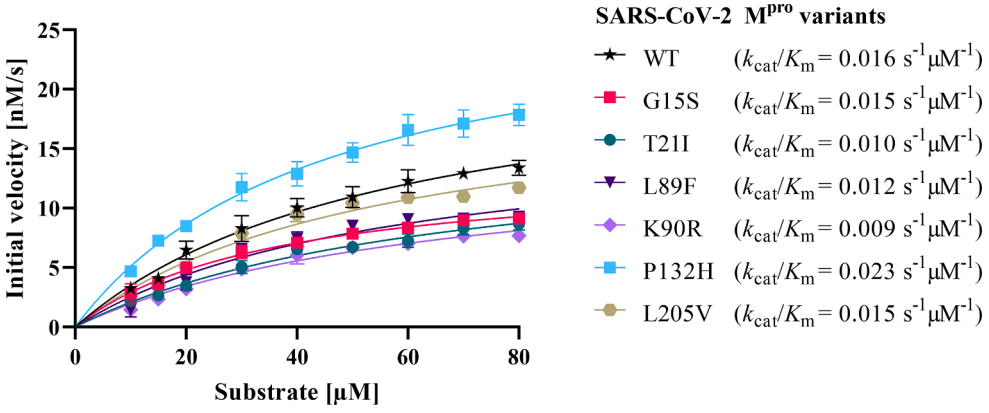


Fig. 3. Michaelis-Menten kinetics of SARS-CoV-2 M^{pro} variants specifying their catalytic efficiency (k_{cat}/K_m).

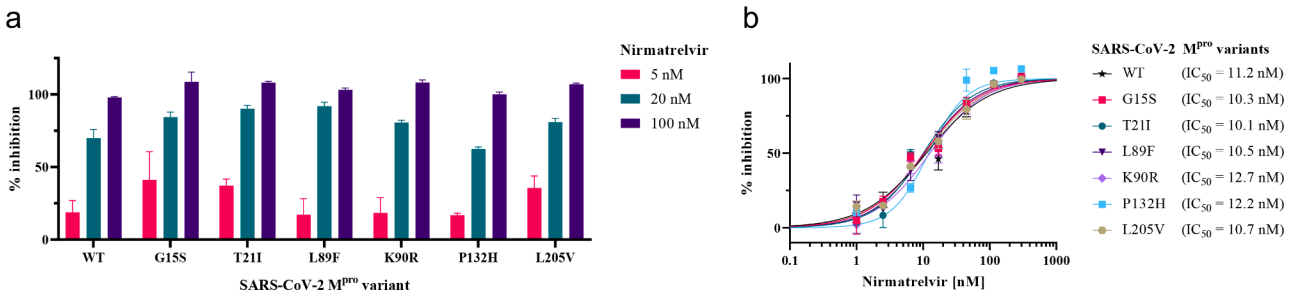


Fig. 4. Inhibition of SARS-CoV-2 M^{pro} variants by nirmatrelvir. (a) Inhibition at 5 nM, 20 nM, and 100 nM. (b) Dose-response curves.

Hence, we selected the six mutations G15S, T21I, L89F, K90R, P132H and L205V for further investigations.

X-ray crystal structures of WT SARS-CoV-2 M^{pro} (e.g. PDB: 6Y2E, 6LU7)[53–54] indicate that the residues G15, T21, K90 and P132 are solvent-exposed, whereas the hydrophobic residues L89 and L205 are buried within the protease. Except for the mutations T21I and P132H, the mutations introduce no major changes in the chemical character of the side-chains, as indicated by low – or, in the case of T21I and P132H, moderate – values of Miyata's distances.[55] The mutations G15S, T21I, L89F and K90R are located in domain I, whereas the mutations P132H and L205V are in domains II and III, respectively (Fig. 2a).[25] To the best of our knowledge, these residues participate neither in the active site nor the allosteric binding sites of SARS-CoV-2 M^{pro} discussed by Günther et al.[56]

WT SARS-CoV-2 M^{pro} and the mutants G15S, T21I, L89F, K90R, P132H and L205V were expressed in *E. coli* and purified. An established Förster resonance electron transfer (FRET) *in vitro* assay of M^{pro} activity[54,57] was employed to determine initial velocities of the proteolytic activity at various substrate concentrations. The data confirmed that all mutants are enzymatically active, which was expected[25,58] as a dysfunctional M^{pro} would prevent replication of SARS-CoV-2. The seven M^{pro} variants exhibited turnover numbers (k_{cat}) between 0.54 and 1.03 s^{-1}, and Michaelis constants (K_m) ranging from 37 to 67 μM (Table S1). The catalytic efficiencies (k_{cat}/K_m) calculated for the mutants (0.009 to 0.023 s^{-1} μM^{-1}) are similar to that of WT M^{pro} (0.016 $s^{-1}\mu M^{-1}$), confirming that all M^{pro} variants are equally competent with regard to their proteolytic activities (Fig. 3, Table S1).

Following the kinetic analysis of the SARS-CoV-2 M^{pro} variants, the clinical candidate nirmatrelvir (also known as PF-07321332; Fig. 1) was used to assess the potential impact of M^{pro} mutations on the drug's efficacy (Fig. 4). The inhibition constant (K_i) of nirmatrelvir against SARS-CoV-2 WT M^{pro} has been reported to be 3.1 nM.[28] Our FRET assay confirmed that nirmatrelvir inhibits the activity of M^{pro} variants at nanomolar inhibitor concentrations. Furthermore, the extent of inhibition was similar across the different protease variants. An initial screening at three selected concentrations showed that the compound displayed below 50% inhibition at 5 nM, over 50% inhibition at 20 nM and fully inhibited the enzymatic activity of all mutants and the WT at 100 nM (Fig. 4**a**). Subsequently, we determined IC_{50} values of nirmatrelvir against the mutants and WT, ranging from 10 nM to 13 nM with overlapping confidence intervals (Fig. 4b, Table S2).

In summary, we identified the currently most prevalent M^{pro} variants (G15S, T21I, L89F, K90R, P132H, L205V) in different lineages of SARS-CoV-2 (C.37 Lambda, B.1.1.318, B.1.2, B.1.351 Beta, B.1.1.529 Omicron, P.2 Zeta) and found that, in a biochemical assay, they are catalytically competent to a similar degree as the wildtype. In addition, we confirmed that nirmatrelvir maintains effective inhibition of all these M^{pro} variants *in vitro*. This suggests that the inhibitory effect of nirmatrelvir and potentially other specific SARS-CoV-2 M^{pro} inhibitors would at present not be compromised for these virus variants. It must be noted, however, that widespread use of M^{pro} inhibitors may challenge SARS-CoV-2 to develop M^{pro} mutations that overcome these inhibitors, as previously experienced for, e.g., HIV protease inhibitors.[59] Despite these challenges, protease inhibitors have revolutionized antiviral treatment for viral infectious diseases, including HIV and HCV.[60] It can thus be expected that M^{pro} inhibitors will have a similar impact on the future development of the COVID-19 pandemic.

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.2022.128629.

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