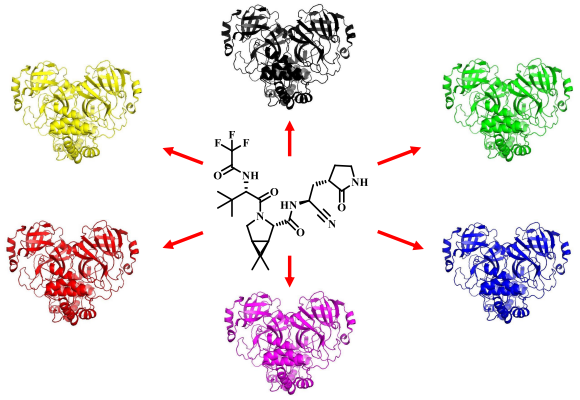
**Main protease mutants of SARS-CoV-2 variants remain susceptible to PF-07321332**

Sven Ullrich, Kasuni B. Ekanayake, Gottfried Otting, Christoph Nitsche*

Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia

*christoph.nitsche@anu.edu.au

**Graphical Abstract**



**Keywords**

SARS-CoV-2, main protease, variants, PF-07321332, inhibitors

**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 PF-07321332. We expressed $M^{pro}$ of five SARS-CoV-2 lineages (C.37 Lambda, B.1.1.318, B.1.2, B.1.351 Beta, P.2 Zeta), each of which carries a strongly prevalent missense mutation (G15S, T21I, L89F, K90R, L205V). Enzyme kinetics showed that these $M^{pro}$ variants are similarly catalytically competent as the wildtype. We show that PF-07321332 has similar potency against the variants as against the wildtype. Our *in vitro* data suggest that the efficacy of specific $M^{pro}$ inhibitors such as PF-07321332 is not compromised in current COVID-19 variants.

Since its emergence in late 2019,[1] COVID-19 has significantly impacted on societies worldwide.[2] More than 5 million deaths have been attributed to COVID-19, with the number of confirmed SARS-CoV-2 infections surpassing 250 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 pronounced genetic variability,[10, 11] it is a common concern that the effectiveness of existing vaccines may be affected by those variants of SARS-CoV-2.[5, 6, 10, 12] At the time of writing, the World Health Organization (WHO) lists four variants of concern (VOC; Alpha, Beta, Gamma, Delta) and two variants of interest (VOI; Lambda, Mu).[13] A possible adjustment of the vaccines to currently circulating lineages of SARS-CoV-2 is being investigated.[14-16] It is clear that the deployment of vaccines 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 and there are currently no approved antiviral drugs specifically designed to target coronaviruses. Only very recently, in November 2021, the orally active drug molnupiravir (MK-4482, EIDD-2801, Lagevrio™) was approved in the United Kingdom. Molnupiravir targets RdRp by acting as a nucleoside analogue prodrug, but was originally developed against different RNA viruses.[26] Lately, the peptidomimetic PF-07321332 (Paxlovid™ as combination drug with ritonavir as booster) has also drawn attention as a potential COVID-19 therapeutic,[27] as the compound has entered phase 3 clinical trials (NCT04960202, NCT05011513, NCT05047601). PF-07321332 is an orally available $M^{pro}$ inhibitor, employing a nitrile warhead to covalently bind the catalytic cysteine residue in the active site of the protease (**Figure 2a**).[27]

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, 28] Inhibition of $M^{pro}$ thus ultimately hinders the assembly of the replication and transcription complexes (RTCs).[25, 29] The protease has a distinct recognition motif, with – in the Schechter-Berger notation[30] – preference for leucine in $P_2$ and especially strong preference for glutamine in $P_1$.[25, 31] Human

host proteases have different substrate specificities and it is therefore anticipated that selective inhibitors have limited off-target effects.[25]

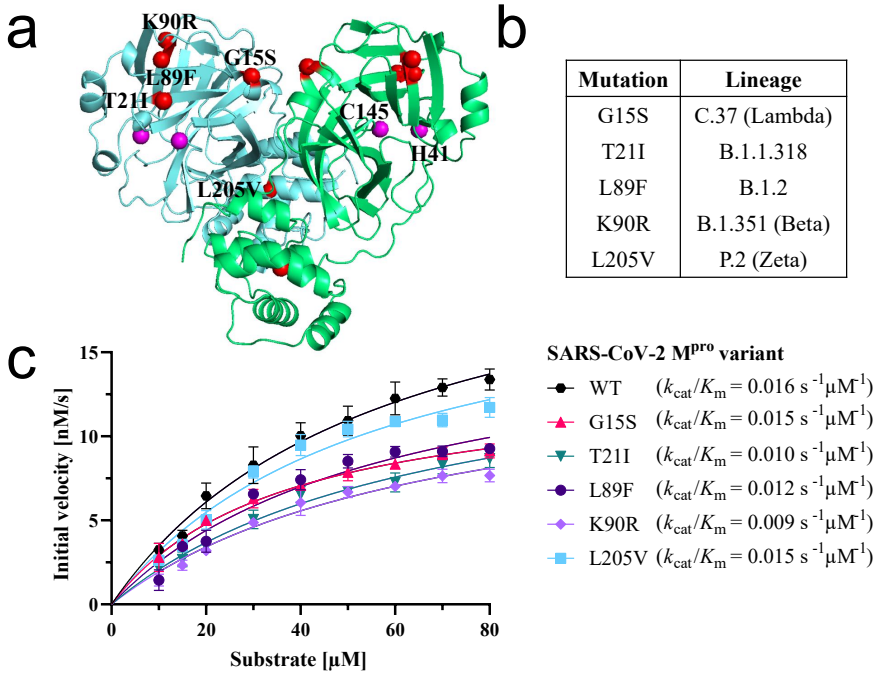
Previous research on SARS-CoV-1 $M^{pro}$ (which is 96% identical in amino acid sequence to SARS-CoV-2 $M^{pro}$)[25] demonstrated that missense point mutations can influence protease activity. Mutants have been identified with slightly enhanced (Ser284, Thr285, Ile286)[32, 33] or slightly or severely reduced catalytic activity (Gly11, Asn28, Ser139, Phe140, Glu166, Asn214, Arg298).[32, 34-38] 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.[35] The present study assesses the $M^{pro}$ mutants of emerging SARS-CoV-2 lineages. We analysed the most widespread amino acid substitutions in SARS-CoV-2 $M^{pro}$, characterized them by enzyme kinetics and assessed their susceptibility to inhibition by PF-07321332.

Utilizing the Outbreak.info database by Scripps Research,[39] which partially operates with data provided by the GISAID Initiative,[40] we performed an analysis of the genomes of SARS-CoV-2 lineages, including the VOC and VOI. The WIV04 sequence (EPI_ISL_402124)[41] acted as wildtype reference genome. Lineage comparison[42] of VOCs and VOIs revealed two missense mutations in the $M^{pro}$ section of the ORF1a/b gene with >20% frequency of occurrance. The mutations are G15S, which is >85% prevalent[43] in the Lambda VOI (or C.37, using PANGO nomenclature)[44] and K90R, which is >95% prevalent[45] in the Beta VOC (B.1.351). The Delta VOC (B.1.617.2), which is the dominant lineage at present,[46] did not display any particularly prevalent (>20%)[42] missense mutations within the $M^{pro}$ part of ORF1a/b at the time of writing. We additionally chose to investigate three additional abundant $M^{pro}$ mutations to cover a larger variety of lineages: T21I, which is >90% prevalent[47] in B.1.1.318, a WHO variant under monitoring (VUM),[13] L89F, which is >95% prevalent[48] in the B.1.2 lineage, and L205V, which is >95% prevalent[49] in the former VOI Zeta (P.2) (**Figure 1b**). Hence, we selected the five mutations G15S, T21I, L89F, K90R and L205V for further investigations.

X-ray crystal structures of wildtype (WT) SARS-CoV-2 $M^{pro}$ (e.g. PDB: 6LU7)[50] indicate that the polar residues G15, T21 and K90 are solvent-exposed, while the hydrophobic residues L89 and L205 are buried within the protease. Except for T21I, the mutations introduce no major changes in the chemical character of the side-chains, as indicated by low, or in the case of T21S moderate, Miyata's distances.[51] The mutations G15S, T21I, L89F and K90R are in domain I, while the mutation L205V is located in domain III (**Figure 1a**).[25]

WT SARS-CoV-2 $M^{pro}$ and the mutants G15S, T21I, L89F, K90R and L205V were expressed in *E. coli* and purified. An established Förster resonance electron transfer (FRET) *in*

*vitro* assay of $M^{pro}$ activity[52] 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, 53] as a dysfunctional $M^{pro}$ would prevent replication of SARS-CoV-2. The six $M^{pro}$ variants exhibited turnover numbers ($k_{cat}$) between 0.54 and 0.95 $s^{-1}$, and Michaelis constants ($K_m$) from 37 to 67 μM (**Table 1**). The catalytic efficiencies ($k_{cat}/K_m$) calculated for the mutants (0.009 to 0.015 $s^{-1}\mu M^{-1}$) 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 (**Figure 1c**, **Table 1**).




| Mutation | Lineage |
|---|---|
| G15S | C.37 (Lambda) |
| T21I | B.1.1.318 |
| L89F | B.1.2 |
| K90R | B.1.351 (Beta) |
| L205V | P.2 (Zeta) |

**Figure 1.** Comparison of $M^{pro}$ mutations and proteolytic activities. (a) X-ray structure of SARS-CoV-2 $M^{pro}$ (PDB: 6LU7)[50] indicating the location of mutations (red) and the catalytic dyad (purple) in the two protomers (cyan, green). (b) List of prevalent $M^{pro}$ mutations and their corresponding SARS-CoV-2 lineage. (c) Michaelis-Menten kinetics of SARS-CoV-2 $M^{pro}$ variants specifying their catalytic efficiency ($k_{cat}/K_m$).

**Table 1.** Michaelis-Menten parameters of SARS-CoV-2 $M^{pro}$ variants.

| Variant | $k_{cat}$ [$s^{-1}$] | $K_m$ [μM] | $k_{cat}/K_m$ [$s^{-1}\mu M^{-1}$] |
|---|---|---|---|
| WT | 0.94 ± 0.08 | 57 ± 9 | 0.016 ± 0.004 |
| G15S | 0.54 ± 0.03 | 37 ± 4 | 0.015 ± 0.002 |
| T21I | 0.64 ± 0.06 | 67 ± 11 | 0.010 ± 0.003 |
| L89F | 0.69 ± 0.08 | 58 ± 13 | 0.012 ± 0.004 |
| K90R | 0.60 ± 0.06 | 67 ± 12 | 0.009 ± 0.003 |
| L205V | 0.82 ± 0.08 | 55 ± 10 | 0.015 ± 0.004 |

Following the kinetic analysis of the SARS-CoV-2 $M^{pro}$ variants, the clinical candidate PF-07321332 (**Figure 2a**) was used to assess the potential impact of $M^{pro}$ mutations on the drug's efficacy. The inhibition constant ($K_i$) of PF-07321332 against SARS-CoV-2 WT $M^{pro}$ has been reported to be 3.1 nM.[27] Our FRET assay confirmed that PF-07321332 inhibits the activity of $M^{pro}$ variants at nanomolar compound concentrations. Furthermore, the extent of inhibition was similar across the different protease variants, with 5 nM compound displaying inhibition below 50%, 20 nM showing inhibition over 50% and 100 nM fully inhibiting the enzymatic activity of all mutants and the WT (**Figure 2b**).

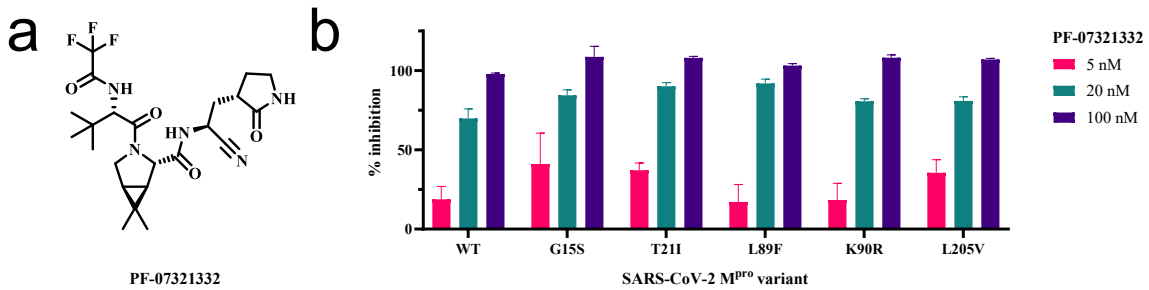



**Figure 2.** Inhibition of SARS-CoV-2 $M^{pro}$ variants. (a) Chemical structure of the inhibitor PF-07321332.[27] (b) *In vitro* inhibition of SARS-CoV-2 $M^{pro}$ variants by PF-07321332.

In summary, we identified the currently most prevalent $M^{pro}$ variants (G15S, T21I, L89F, K90R, L205V) in different lineages of SARS-CoV-2 (C.37 Lambda, B.1.1.318, B.1.2, B.1.351 Beta, P.2 Zeta) and experimentally confirmed that they are equally potent as the wildtype. In addition, we confirmed that PF-07321332 maintains effective inhibition of all these $M^{pro}$ variants. This gives hope that SARS-CoV-2 $M^{pro}$ inhibitors at present would not be negatively affected by 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.[54] Despite these challenges, protease inhibitors have revolutionized antiviral treatment for viral infectious diseases, including HIV and HCV.[55] It can thus be expected that $M^{pro}$ inhibitors will have a similar impact on the future development of the COVID-19 pandemic.

**Acknowledgements**

C.N. thanks the Australian Research Council (ARC) for a Discovery Early Career Research Award (DE190100015) and Discovery Project funding (DP200100348). G.O. thanks the ARC for a Laureate Fellowship (FL170100019) and acknowledges support by the ARC Centre of Excellence for Innovations in Peptide & Protein Science (CE200100012). This study was supported by a RAMR (MAWA) grant awarded to S.U. and C.N.

**Competing Interest Declaration**

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.

**Author Information**

Sven Ullrich, ORCID: 0000-0003-4184-7024

Kasuni B. Ekanayake, ORCID: 0000-0002-3078-5895

Gottfried Otting, ORCID: 0000-0002-0563-0146

Christoph Nitsche, ORCID: 0000-0002-3704-2699

**References**

(1) Zhu, N.; Zhang, D.; Wang, W.; Li, X.; Yang, B.; Song, J.; Zhao, X.; Huang, B.; Shi, W.; Lu, R.; et al. A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* **2020**, *382* (8), 727–733. DOI: 10.1056/NEJMoa2001017.

(2) Hiscott, J.; Alexandridi, M.; Muscolini, M.; Tassone, E.; Palermo, E.; Soultsioti, M.; Zevini, A. The global impact of the coronavirus pandemic. *Cytokine Growth Factor Rev.* **2020**, *53*, 1–9. DOI: 10.1016/j.cytogfr.2020.05.010.

(3) Dong, E.; Du, H.; Gardner, L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect. Dis.* **2020**, *20* (5), 533–534. DOI: 10.1016/S1473-3099(20)30120-1.

(4) Zhang, J.; Zeng, H.; Gu, J.; Li, H.; Zheng, L.; Zou, Q. Progress and prospects on vaccine development against SARS-CoV-2. *Vaccines* **2020**, *8* (2). DOI: 10.3390/vaccines8020153.

(5) Subbarao, K. The success of SARS-CoV-2 vaccines and challenges ahead. *Cell Host Microbe* **2021**, *29* (7), 1111–1123. DOI: 10.1016/j.chom.2021.06.016.

(6) Tregoning, J. S.; Flight, K. E.; Higham, S. L.; Wang, Z.; Pierce, B. F. Progress of the COVID-19 vaccine effort: viruses, vaccines and variants versus efficacy, effectiveness and escape. *Nat. Rev. Immunol.* **2021**, *21* (10), 626–636. DOI: 10.1038/s41577-021-00592-1.

(7) Le, T. T.; Cramer, J. P.; Chen, R.; Mayhew, S. Evolution of the COVID-19 vaccine development landscape. *Nat. Rev. Drug Discov.* **2020**, *19* (10), 667–668. DOI: 10.1038/d41573-020-00151-8.

(8) Teijaro, J. R.; Farber, D. L. COVID-19 vaccines: modes of immune activation and future challenges. *Nat. Rev. Immunol.* **2021**, *21* (4), 195–197. DOI: 10.1038/s41577-021-00526-x.

(9) Dupont, L.; Snell, L. B.; Graham, C.; Seow, J.; Merrick, B.; Lechmere, T.; Maguire, T. J. A.; Hallett, S. R.; Pickering, S.; Charalampous, T.; et al. Neutralizing antibody activity in convalescent sera from infection in humans with SARS-CoV-2 and variants of concern. *Nat. Microbiol.* **2021**, *6* (11), 1433–1442. DOI: 10.1038/s41564-021-00974-0.

(10) Tao, K.; Tzou, P. L.; Nouhin, J.; Gupta, R. K.; de Oliveira, T.; Kosakovsky Pond, S. L.; Fera, D.; Shafer, R. W. The biological and clinical significance of emerging SARS-CoV-2 variants. *Nat. Rev. Genet.* **2021**. DOI: 10.1038/s41576-021-00408-x.

(11) Harvey, W. T.; Carabelli, A. M.; Jackson, B.; Gupta, R. K.; Thomson, E. C.; Harrison, E. M.; Ludden, C.; Reeve, R.; Rambaut, A.; Peacock, S. J.; et al. SARS-CoV-2 variants, spike mutations and immune escape. *Nat. Rev. Microbiol.* **2021**, *19* (7), 409–424. DOI: 10.1038/s41579-021-00573-0.

(12) Tatsi, E.-B.; Filippatos, F.; Michos, A. SARS-CoV-2 variants and effectiveness of vaccines: a review of current evidence. *Epidemiol. Infect.* **2021**, 1–24. DOI: 10.1017/s0950268821002430.

(13) World Health Organization. *Tracking SARS-CoV-2 variants*. 2021. https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/ (accessed 7 Nov 2021).

(14) Prévost, J.; Finzi, A. The great escape? SARS-CoV-2 variants evading neutralizing responses. *Cell Host Microbe* **2021**, *29* (3), 322–324. DOI: 10.1016/j.chom.2021.02.010.

(15) Al-Zyoud, W.; Haddad, H. Dynamics prediction of emerging notable spike protein mutations in SARS-CoV-2 implies a need for updated vaccines. *Biochimie* **2021**, *191*, 91–103. DOI: 10.1016/j.biochi.2021.08.011.

(16) Cevik, M.; Grubaugh, N. D.; Iwasaki, A.; Openshaw, P. COVID-19 vaccines: keeping pace with SARS-CoV-2 variants. *Cell* **2021**, *184* (20), 5077–5081. DOI: 10.1016/j.cell.2021.09.010.

(17) Grubaugh, N. D.; Hodcroft, E. B.; Fauver, J. R.; Phelan, A. L.; Cevik, M. Public health actions to control new SARS-CoV-2 variants. *Cell* **2021**, *184* (5), 1127–1132. DOI: 10.1016/j.cell.2021.01.044.

(18) Viana, J.; van Dorp, C. H.; Nunes, A.; Gomes, M. C.; van Boven, M.; Kretzschmar, M. E.; Veldhoen, M.; Rozhnova, G. Controlling the pandemic during the SARS-CoV-2 vaccination rollout. *Nat. Commun.* **2021**, *12* (1), 3674. DOI: 10.1038/s41467-021-23938-8.

(19) Hobman, T. C.; Murgolo, N.; Therien, A. G.; Howell, B.; Klein, D.; Koeplinger, K.; Lieberman, L. A.; Adam, G. C.; Flynn, J.; McKenna, P.; et al. SARS-CoV-2 tropism, entry, replication, and propagation: considerations for drug discovery and development. *PLOS Pathog.* **2021**, *17* (2), e1009225. DOI: 10.1371/journal.ppat.1009225.

(20) Drosten, C.; Günther, S.; Preiser, W.; van der Werf, S.; Brodt, H.-R.; Becker, S.; Rabenau, H.; Panning, M.; Kolesnikova, L.; Fouchier, R. A. M.; et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* **2003**, *348* (20), 1967–1976. DOI: 10.1056/NEJMoa030747.

(21) Hilgenfeld, R.; Peiris, M. From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. *Antivir. Res.* **2013**, *100* (1), 286–295. DOI: 10.1016/j.antiviral.2013.08.015.

(22) Zhu, W.; Chen, C. Z.; Gorshkov, K.; Xu, M.; Lo, D. C.; Zheng, W. RNA-dependent RNA polymerase as a target for COVID-19 drug discovery. *SLAS Discov.* **2020**, *25* (10), 1141–1151. DOI: 10.1177/2472555220942123.

(23) Spratt, A. N.; Gallazzi, F.; Quinn, T. P.; Lorson, C. L.; Sönnerborg, A.; Singh, K. Coronavirus helicases: attractive and unique targets of antiviral drug-development and therapeutic patents. *Expert Opin. Ther. Pat.* **2021**, *31* (4), 339–350. DOI: 10.1080/13543776.2021.1884224.

(24) Mahmoudvand, S.; Shokri, S. Interactions between SARS coronavirus 2 papain-like protease and immune system: A potential drug target for the treatment of COVID-19. *Scand. J. Immunol.* **2021**, *94* (4), e13044. DOI: 10.1111/sji.13044.

(25) Ullrich, S.; Nitsche, C. The SARS-CoV-2 main protease as drug target. *Bioorg. Med. Chem. Lett.* **2020**, *30* (17), 127377. DOI: 10.1016/j.bmcl.2020.127377.

(26) Painter, G. R.; Natchus, M. G.; Cohen, O.; Holman, W.; Painter, W. P. Developing a direct acting, orally available antiviral agent in a pandemic: the evolution of molnupiravir as a potential treatment for COVID-19. *Curr. Opin. Virol.* **2021**, *50*, 17–22. DOI: 10.1016/j.coviro.2021.06.003.

(27) Owen, D. R.; Allerton, C. M. N.; Anderson, A. S.; Aschenbrenner, L.; Avery, M.; Berritt, S.; Boras, B.; Cardin, R. D.; Carlo, A.; Coffman, K. J.; et al. An oral SARS-CoV-2 $M^{pro}$ inhibitor clinical candidate for the treatment of COVID-19. *Science* **2021**, eabl4784. DOI: 10.1126/science.abl4784.

(28) Kim, D.; Lee, J.-Y.; Yang, J.-S.; Kim, J. W.; Kim, V. N.; Chang, H. The architecture of SARS-CoV-2 transcriptome. *Cell* **2020**, *181* (4), 914–921.e910. DOI: 10.1016/j.cell.2020.04.011.

(29) Hartenian, E.; Nandakumar, D.; Lari, A.; Ly, M.; Tucker, J. M.; Glaunsinger, B. A. The molecular virology of coronaviruses. *J. Biol. Chem.* **2020**, *295* (37), 12910–12934. DOI: 10.1074/jbc.REV120.013930.

(30) Schechter, I.; Berger, A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27* (2), 157–162. DOI: 10.1016/s0006-291x(67)80055-x.

(31) Rut, W.; Groborz, K.; Zhang, L.; Sun, X.; Zmudzinski, M.; Pawlik, B.; Wang, X.; Jochmans, D.; Neyts, J.; Młynarski, W.; et al. SARS-CoV-2 $M^{pro}$ inhibitors and activity-based probes for patient-sample imaging. *Nat. Chem. Biol.* **2020**, *17* (2), 222–228. DOI: 10.1038/s41589-020-00689-z.

(32) Shi, J.; Song, J. The catalysis of the SARS 3C-like protease is under extensive regulation by its extra domain. *FEBS Journal* **2006**, *273* (5), 1035–1045. DOI: 10.1111/j.1742-4658.2006.05130.x.

(33) Fraternali, F.; Lim, L.; Shi, J.; Mu, Y.; Song, J. Dynamically-driven enhancement of the catalytic machinery of the SARS 3C-like protease by the S284-T285-I286/A mutations on the extra domain. *PLOS One* **2014**, *9* (7), e101941. DOI: 10.1371/journal.pone.0101941.

(34) Chen, S.; Hu, T.; Zhang, J.; Chen, J.; Chen, K.; Ding, J.; Jiang, H.; Shen, X. Mutation of Gly-11 on the dimer interface results in the complete crystallographic dimer dissociation of severe acute respiratory syndrome coronavirus 3C-like protease: crystal structure with molecular dynamics simulations. *J. Biol. Chem.* **2008**, *283* (1), 554–564. DOI: 10.1074/jbc.M705240200.

(35) Shi, J.; Sivaraman, J.; Song, J. Mechanism for controlling the dimer-monomer switch and coupling dimerization to catalysis of the severe acute respiratory syndrome coronavirus 3C-like protease. *J. Virol.* **2008**, *82* (9), 4620–4629. DOI: 10.1128/jvi.02680-07.

(36) Barrila, J.; Gabelli, S. B.; Bacha, U.; Amzel, L. M.; Freire, E. Mutation of Asn28 disrupts the dimerization and enzymatic activity of SARS $3CL^{pro}$. *Biochemistry* **2010**, *49* (20), 4308–4317. DOI: 10.1021/bi1002585.

(37) Hu, T.; Zhang, Y.; Li, L.; Wang, K.; Chen, S.; Chen, J.; Ding, J.; Jiang, H.; Shen, X. Two adjacent mutations on the dimer interface of SARS coronavirus 3C-like protease cause different conformational changes in crystal structure. *Virology* **2009**, *388* (2), 324–334. DOI: 10.1016/j.virol.2009.03.034.

(38) Cheng, S.-C.; Chang, G.-G.; Chou, C.-Y. Mutation of Glu-166 blocks the substrate-induced dimerization of SARS coronavirus main protease. *Biophys. J.* **2010**, *98* (7), 1327–1336. DOI: 10.1016/j.bpj.2009.12.4272.

(39) Mullen, J. L.; Tsueng, G.; Latif, A. A.; Alkuzweny, M.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - a standardized, open-source database of COVID-19 resources and epidemiology data* 2020. https://outbreak.info/ (accessed 7 Nov 2021).

(40) Elbe, S.; Buckland-Merrett, G. Data, disease and diplomacy: GISAID's innovative contribution to global health. *Glob. Chall.* **2017**, *1* (1), 33–46. DOI: 10.1002/gch2.1018.

(41) Zhukova, A.; Blassel, L.; Lemoine, F.; Morel, M.; Voznica, J.; Gascuel, O. Origin, evolution and global spread of SARS-CoV-2. *C. R. Biol.* **2021**, *344* (1), 57–75. DOI: 10.5802/crbiol.29.

(42) Abdel Latif, A.; Mullen, J. L.; Alkuzweny, M.; Tsueng, G.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - lineage comparison*. 2021. https://outbreak.info/compare-lineages (accessed 8 Nov 2021).

(43) Abdel Latif, A.; Mullen, J. L.; Alkuzweny, M.; Tsueng, G.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - ORF1a:G3278S mutation report*. 2021. https://outbreak.info/situation-reports?pango&muts=ORF1a%3AG3278S (accessed 8 Nov 2021).
(44) Rambaut, A.; Holmes, E. C.; O'Toole, Á.; Hill, V.; McCrone, J. T.; Ruis, C.; du Plessis, L.; Pybus, O. G. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. *Nat. Microbiol.* **2020**, *5* (11), 1403–1407. DOI: 10.1038/s41564-020-0770-5.
(45) Abdel Latif, A.; Mullen, J. L.; Alkuzweny, M.; Tsueng, G.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - ORF1a:K3353R mutation report*. 2021. https://outbreak.info/situation-reports?pango&muts=ORF1a%3AK3353R (accessed 8 Nov 2021).
(46) He, X.; He, C.; Hong, W.; Zhang, K.; Wei, X. The challenges of COVID-19 Delta variant: prevention and vaccine development. *MedComm* **2021**. DOI: 10.1002/mco2.95.
(47) Abdel Latif, A.; Mullen, J. L.; Alkuzweny, M.; Tsueng, G.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - ORF1a:T3284I mutation report*. 2021. https://outbreak.info/situation-reports?pango&muts=ORF1a%3AT3284I (accessed 8 Nov 2021).
(48) Abdel Latif, A.; Mullen, J. L.; Alkuzweny, M.; Tsueng, G.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - ORF1a:L3352F mutation report*. 2021. https://outbreak.info/situation-reports?pango&muts=ORF1a%3AL3352F (accessed 8 Nov 2021).
(49) Abdel Latif, A.; Mullen, J. L.; Alkuzweny, M.; Tsueng, G.; Cano, M.; Haag, E.; Zhou, J.; Zeller, M.; Hufbauer, E.; Matteson, N.; et al. *Outbreak.info - ORF1a:L3468V mutation report*. 2021. https://outbreak.info/situation-reports?pango&muts=ORF1a%3AL3468V (accessed 8 Nov 2021).
(50) Jin, Z.; Du, X.; Xu, Y.; Deng, Y.; Liu, M.; Zhao, Y.; Zhang, B.; Li, X.; Zhang, L.; Peng, C.; et al. Structure of $M^{pro}$ from SARS-CoV-2 and discovery of its inhibitors. *Nature* **2020**, *582* (7811), 289–293. DOI: 10.1038/s41586-020-2223-y.
(51) Miyata, T.; Miyazawa, S.; Yasunaga, T. Two types of amino acid substitutions in protein evolution. *J. Mol. Evol.* **1979**, *12* (3), 219–236. DOI: 10.1007/bf01732340.
(52) Ullrich, S.; Sasi, V. M.; Mahawaththa, M. C.; Ekanayake, K. B.; Morewood, R.; George, J.; Shuttleworth, L.; Zhang, X.; Whitefield, C.; Otting, G.; et al. Challenges of short substrate

analogues as SARS-CoV-2 main protease inhibitors. *Bioorg. Med. Chem. Lett.* **2021**, *50*, 128333. DOI: 10.1016/j.bmcl.2021.128333.

(53) Zeldovich, K. B.; Chen, P.; Shakhnovich, E. I. Protein stability imposes limits on organism complexity and speed of molecular evolution. *Proc. Natl. Acad. Sci. USA* **2007**, *104* (41), 16152–16157. DOI: 10.1073/pnas.0705366104.

(54) Wensing, A. M. J.; van Maarseveen, N. M.; Nijhuis, M. Fifteen years of HIV protease inhibitors: raising the barrier to resistance. *Antivir. Res.* **2010**, *85* (1), 59–74. DOI: 10.1016/j.antiviral.2009.10.003.

(55) Agbowuro, A. A.; Huston, W. M.; Gamble, A. B.; Tyndall, J. D. A. Proteases and protease inhibitors in infectious diseases. *Med. Res. Rev.* **2018**, *38* (4), 1295–1331. DOI: 10.1002/med.21475.