Supporting Information

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

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Abbreviations

CI	Confidence interval
CoV	Coronavirus
DABCYL	4-((4-(Dimethylamino)phenyl)azo)benzoic acid
DTT	Dithiothreitol
EDANS	5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid
EDTA	Ethylenediaminetetraacetic acid
FRET	Förster resonance electron transfer
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Half maximal inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Lysogeny broth
M^{pro}	Main protease
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulphate
TB	Terrific broth
UHPLC	Ultra high-performance liquid chromatography
WT	Wildtype

Plasmid construction

The SARS-CoV-2 M^{pro} gene[1] was cloned between the *NdeI* and *XhoI* sites of the T7 vector pET-47b (+). The construct contains the M^{pro} cleavage-site (SAVLQ↓SGFRK; arrow indicating the cleavage site) at the N-terminus and a modified PreScission cleavage site (SGVTFQ↓GP) followed by a His_6-tag at the C-terminus. Point mutagenesis to incorporate an amino acid of choice at selected sites was performed using overlapping primers. All plasmid constructions and mutagenesis were conducted with a RQ-SLIC and QuikChange protocol using mutant T4 DNA polymerase.[2]

Protein expression

To produce WT M^{pro} and mutants, *E. coli* BL21 DE3 cells were transformed with the respective pET-47b (+)-M^{pro} plasmid and the cells were grown at 37 °C in LB medium containing 50 mg/L kanamycin. Overnight cultures were inoculated into fresh TB medium (1:100 dilution) supplemented with 50 mg/L kanamycin. The cells were grown at 37 °C to an OD_{600} of 0.6 to 1.0. Expression was initiated by the addition of IPTG to a final concentration of 1 mM. Protein expression was conducted by incubation at room temperature overnight.

Protein purification

Cells were harvested by centrifugation at 5,000 *g* for 15 min. Following resuspension in buffer A (50 mM Tris-HCl, pH 7.5, 300 mM NaCl), the cells were lysed using an Emulsiflex C5 (Avestin, Canada) with two passes using 10,000–15,000 psi. The cell lysates were centrifuged for 1 h at 30,000 *g*. The supernatant was loaded onto a 1 mL His GraviTrap TALON® column (Cytiva, United States). The column was washed with 20 column volumes buffer B (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM imidazole) and the protein was eluted with 5 column volumes buffer C (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 300 mM imidazole). The fractions were analysed by 12% SDS-PAGE. Following purification, the buffer was exchanged to buffer D (20 mM HEPES-KOH, pH 7.0, 150 mM NaCl, 1 mM DTT, 1 mM EDTA) using an Amicon ultrafiltration centrifugal tube (Merck Millipore, United States) with a molecular weight cut-off of 10 kDa. All samples were analysed by mass spectrometry using an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Scientific, United States) coupled with an UltiMate S4 3000 UHPLC (Thermo Scientific, United States). Protein concentrations were determined by measuring the absorbance at 280 nm, using $\varepsilon = 33{,}640\ L \cdot M^{-1} \cdot cm^{-1}$.

FRET assay

The SARS-CoV-2 M^{pro} FRET assay employed for the kinetic evaluation and the inhibitory assessment was based on the SARS-CoV-1 M^{pro} assay by Zhu et al.[3] and the SARS-CoV-2 M^{pro} assay by Zhang et al.[1] Specific conditions were adapted from Ullrich et al.[4] The assay was carried out in black 96-well polypropylene plates with U-bottom (Greiner Bio-One, Austria), using a buffer of 20 mM Tris-HCl pH 7.3, 100 mM NaCl, 1 mM EDTA, 1 mM DTT. The peptide DABCYL-KTSAVLQ↓SGFRKM-E(EDANS)-NH_2 (Mimotopes, Australia) served as FRET substrate. The concentration of the recombinant SARS-CoV-2 M^{pro} variants amounted to 25 nM. The inhibitor nirmatrelvir[5] – also known as PF-07321332 – (MedChemExpress, United States; Batch HY-138687-116180)[6] was used to assess the inhibition of the proteases. For the kinetic assessment, FRET substrate concentrations varied between 10 µM and 80 µM, and the inhibition assays were conducted with a FRET substrate concentration of 25 µM. When inhibitor was used, it was incubated with the protease for 10 min at 37 °C. The enzymatic reaction was initiated by the addition of FRET substrate and monitored at 37 °C for 5 min at λ_{em} = 490 nm with an excitation wavelength of λ_{ex} = 340 nm, using an Infinite 200 PRO M Plex fluorophotometer (Tecan, Switzerland). Kinetic measurements were performed in duplicate and inhibitory measurements in triplicate. 100% enzymatic activity was defined as the initial velocity of the control reactions and % inhibition was calculated accordingly. Fluorescence intensity was converted to cleaved substrate per unit time with an EDANS calibration curve.[7] The data obtained were analyzed and visualized with Prism 9.3 (GraphPad Software, United States). For IC_{50} determination, bottom and top values of the sigmoidal graph fit were constrained to 0% and 100%, respectively.

Amino acid sequence of SARS-CoV-2 M^{pro} WT

SGFRKMAFPSGKVEGCMVQVTCGTTTLNGLWLDDVVYCPRHVICTSEDMLNPNYE
DLLIRKSNHNFLVQAGNVQLRVIGHSMQNCVLKLKVDTANPKTPKYKFVRIQPGQT
FSVLACYNGSPSGVYQCAMRPNFTIKGSFLNGSCGSVGFNIDYDCVSFCYMHHMELP
TGVHAGTDLEGNFYGPFVDRQTAQAAGTDTTITVNVLAWLYAAVINGDRWFLNRF
TTTLNDFNLVAMKYNYEPLTQDHVDILGPLSAQTGIAVLDMCASLKELLQNGMNGR
TILGSALLEDEFTPFDVVRQCSGVTFQ

Table S1. Michaelis-Menten parameters of SARS-CoV-2 M^{pro} variants. 95% CI in brackets.

Variant	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$s^{-1}\mu M^{-1}$]
WT	0.94 (0.77–1.11)	57 (38–76)	0.016 (0.008–0.024)
G15S	0.54 (0.49–0.59)	37 (29–45)	0.015 (0.010–0.020)
T21I	0.64 (0.51–0.77)	67 (43–91)	0.010 (0.005–0.015)
L89F	0.69 (0.87–0.51)	58 (30–86)	0.012 (0.003–0.021)
K90R	0.60 (0.46–0.74)	67 (40–94)	0.009 (0.003–0.015)
P132H	1.03 (0.90–1.16)	45 (33–57)	0.023 (0.014–0.032)
L205V	0.82 (0.65–0.99)	55 (34–76)	0.015 (0.006–0.024)

Table S2. IC_{50} values of nirmatrelvir against SARS-CoV-2 M^{pro} variants. 95% CI in brackets.

Variant	IC_{50} [nM]
WT	11.2 (8.5–14.7)
G15S	10.3 (8.4–12.6)
T21I	10.1 (8.2–12.4)
L89F	10.5 (9.0–12.4)
K90R	12.7 (10.4–15.4)
P132H	12.2 (9.6–15.3)
L205V	10.7 (9.2–12.4)

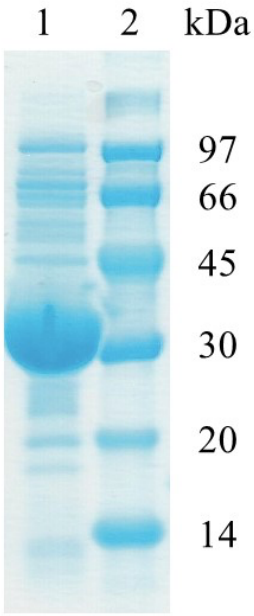

Figure S1. 12% SDS-PAGE images of WT SARS-CoV-2 M^{pro}. Lane 1 corresponds to the purified protein and lane 2 displays protein markers.

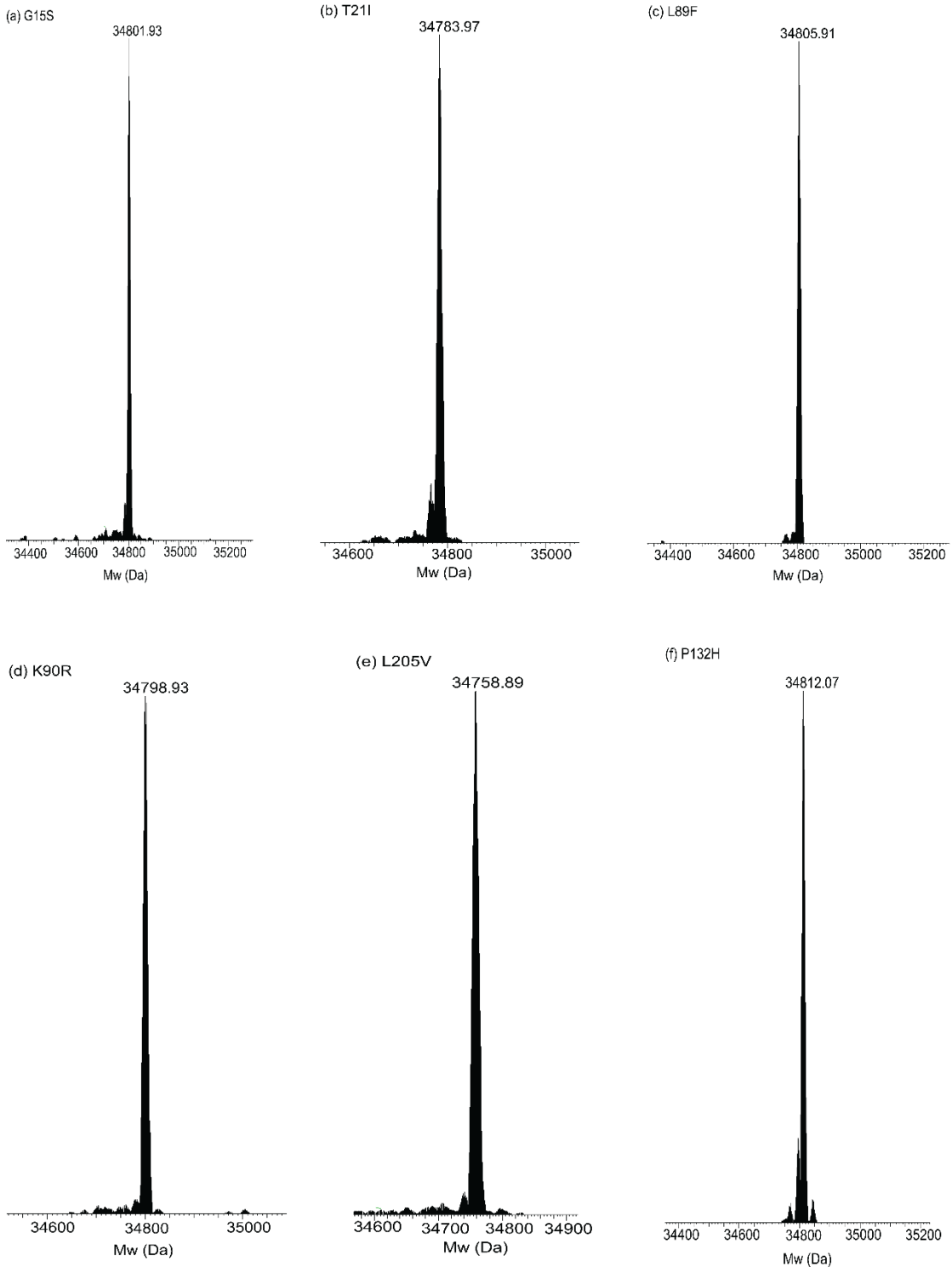

Figure S2. Intact protein mass spectrometric analysis of mutants of SARS-CoV-2 M^{pro}. Calculated molecular weights in brackets. (a) G15S (34803.77 Da). (b) T21I (34785.80 Da). (c) L89F (34807.76 Da). (d) K90R (34801.76 Da). (e) L205V (34759.72 Da). (f) P132H (34813.77 Da).

References

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