

Article

13b-K and Nirmatrelvir Resistance Mutations of SARS-CoV-2 Main Protease: Structural, Biochemical, and Biophysical Characterization of Free Enzymes and Inhibitor Complexes

Haifa El Kilani ^{1,2,*}, Xinyuanyuan Sun ^{1,†}, Mohamed Fouad Ibrahim ¹, Ute Curth ³ and Rolf Hilgenfeld ^{1,2}

¹ Institute of Molecular Medicine, University of Lübeck, 23562 Lübeck, Germany

² German Center for Infection Research (DZIF), Hamburg-Lübeck-Borstel-Riems Site, University of Lübeck, 23562 Lübeck, Germany

³ Institute for Biophysical Chemistry, Hannover Medical School, 30625 Hannover, Germany

* Correspondence: haifa.elkilani@uni-luebeck.de

† These authors contributed equally to this work.

Abstract

The SARS-CoV-2 main protease (M^{Pro}) is a well-established target for antiviral drug development. One such inhibitor, nirmatrelvir, in combination with ritonavir as a booster, has already been introduced into the market, under the name Paxlovid. However, being an RNA virus, SARS-CoV-2 is prone to the emergence of resistance mutations. A number of such mutations have been characterized, although they have not yet been shown to play a significant role in clinical settings; these include S144A, E166V, H172Y, and Q189K. We recombinantly produced these mutants and studied the corresponding proteins using X-ray crystallography, enzymology, and biophysical approaches. We discuss the potential of each mutant to lead to a widespread nirmatrelvir resistance scenario. We also demonstrate that one of our own inhibitors (13b-K), while showing some degree of cross-resistance with nirmatrelvir, exhibits much higher inhibitory activity against the M^{Pro} carrying the E166V mutation.

Keywords: X-ray crystallography; SARS-CoV-2; COVID-19; main protease; drug-resistance mutations; analytical ultracentrifugation



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1. Introduction

The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Wuhan in early 2020 [1] triggered intensive global research efforts, seeking to develop effective vaccines or drug candidates for the prevention or treatment of COVID-19.

The RNA-dependent RNA polymerase (RdRp), nonstructural protein 5 (Nsp5), and the main protease (M^{Pro}, also called 3C-like protease, 3CL^{Pro}) are the most important targets for drug design against SARS-CoV-2 [2]. All three enzymes are relatively conserved among coronaviruses and play a crucial role in the viral life cycle [3,4].

M^{Pro} is a homodimeric cysteine protease; each monomer comprises three domains, domains I, II, and III (residues 8–101, 102–184, and 201–306, respectively) [5–7]. Domains I and II contain the catalytic site (His⁴¹/Cys¹⁴⁵) of the enzyme and the most conserved and essential residues for substrate binding, His41, Phe140, Ser144, Cys145, His163, Glu166, and His172. These residues contribute to the strong binding of the substrate to the S1-S4 specificity sites and to the dimerization of the protein, via essential hydrogen bonds and salt bridges from one monomer to the N-terminal residues of the adjacent monomer

(“N-finger”; most importantly, Ser1 of the adjacent monomer (Ser1*)). As the substrate enters the substrate-binding pocket, the scissile peptide bond of P1'/P1 is nucleophilically attacked by the thiolate of Cys145, resulting in the formation of a tetrahedral transition state. This hemithioketal intermediate is stabilized by the oxyanion hole, which is formed by the backbone amide NH groups of Gly143, Ser144, and Cys145. The stabilization of this transition state lowers the activation energy of the reaction.

In 2021, the Food and Drug Administration of the United States (FDA) [8] provisionally authorized three antiviral drugs for the treatment of mild-to-moderate COVID-19 [2]: remdesivir [9,10] and molnupiravir [11–13], which target the RdRp, and Paxlovid [14], which targets the M^{Pro}. Paxlovid is a combination of the M^{Pro} inhibitor nirmatrelvir and the FDA-approved HIV protease inhibitor ritonavir. In this combination, ritonavir is used as a booster to slow down the metabolism of nirmatrelvir by the human liver cytochrome P450 3A4 (CYP3A4) [14].

Treatment with Paxlovid has been reported to show very good clinical efficacy compared to treatment with placebos, lowering the rates of hospitalization and death by 89% [15].

The genome sequences of naturally occurring nirmatrelvir-resistant SARS-CoV-2 variants, such as those encoding M^{Pro} mutants H172Y, E166V, Q189K, and S144A, have been deposited in the Global Initiative on Sharing Avian Influenza Data (GISAID) database [16]. Although these mutants have only been detected at very low frequencies (35–200 viruses; data as of 7 July 2025), several biochemical studies [17–19] have reported a reduction in the inhibitory activity of nirmatrelvir due to these drug resistance mutations.

To investigate the effects of these mutations on M^{Pro} conformation and inhibitor binding, we determined the crystal structures of the H172Y, E166V, Q189K, and S144A M^{Pro} free enzymes and their complexes with our α -ketoamide M^{Pro} inhibitor 13b-K [20,21], as well as with nirmatrelvir.

2. Materials and Methods

2.1. Compound Synthesis

PF-07321332 (nirmatrelvir) was custom-synthesized by Tocris (Bio-Techne, Wiesbaden, Germany), following the protocol in [20].

2.2. Cloning and Recombinant Protein Production

A gene encoding the SARS-CoV-2 main protease (M^{Pro} wild type, GenBank reference: MN908947.3 (ORF1ab polyprotein residues 3264–3569)) with *E. coli* codon usage was produced by MWG Eurofins. The amplified DNA fragment was digested with the restriction enzymes *Bam*HI and *Xho*I and then ligated into the plasmid pGEX-6P-1. The E166V-SARS-CoV-2 M^{Pro} point mutation was generated by Phusion site-directed mutagenesis PCR (SDM). The PCR reaction solutions were mixed with the Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, MA, USA), using 5' pre-phosphorylated primers (E166V-forward ATGCACCACATGGTGTGCGCGACTG and E166V-reverse ATAGCAGAAGCTAACGCAATCGTAG).

The H172Y, Q189K, and S144A mutations were inserted by overlap extension-PCR reaction. A pair of unique primers—H172Y_forward (ACTGGTGTATATGCCGGGACG-GACT), H172Y_reverse (AGTCCGTCCCGGCATATACACCAGT); Q189K_forward (TTGTC-GATCGCAAAACAGCCCCAA), Q189K_reverse (TTGGGCTGTTTTGCGATCGACAA); and S144A_forward (TTCCTTAATGGCGCGTGTGGTTCGGT), S144A_reverse (ACCGAACC ACACGCGCCATTAAGGAA)—was designed. The first PCR reaction was performed to generate two splice fragments containing a 5' overhang. The WT M^{Pro} coding gene with *Bam*HI and *Xho*I sites was amplified from the M^{Pro} construct described previously [1]

and was used as a template. The second PCR joined these two spliced fragments in order to generate the PCR product encoding the H172Y, Q189K, and S144A mutated M^{Pro}s, including the cleavage sites of the restriction enzymes for cloning into the vector pGEX-6p-1 (GE Healthcare, Chicago, IL, USA). The amplified PCR product was digested with BamHI and XhoI and ligated into the vector pGEX-6p-1 digested with the same restriction enzymes. The gene sequence of the M^{Pro} was verified by sequencing (MWG Eurofins, Ebersberg, Germany).

For recombinant protein production, the SARS-CoV-2 M^{Pro} PGEx6p-1 plasmid was transformed into BL21 (DE3) competent cells and the protein was produced as described before [20].

2.3. Determination of Protein Stability of SARS-CoV-2 M^{Pro} WT, H172Y, E166V, Q189K, and S144A Using Nano-Differential Scanning Fluorimetry (nanoDSF)

Thermal shift assays of the SARS-CoV-2 M^{Pro} WT and the mutants were carried out using the nanoDSF method as implemented in Prometheus NT.48 (NanoTemper Technologies, Munich, Germany). The nanoDSF method is based on the autofluorescence of tryptophan (and tyrosine) residues to monitor protein unfolding. When the temperature increases, the protein will unfold and the hydrophobic residues of the protein are exposed; the ratio of autofluorescence at wavelengths 330 nm and 350 nm will change. The first derivative of 350/330 nm can be used to determine the melting temperature (T_m). Here, 30 μ M of the WT or mutant protein was diluted in a final volume of 15 μ L reaction buffer containing 20 mM HEPES pH7.0, 120 mM NaCl, 0.4 mM EDTA, 4 mM DTT, and 20% glycerol. Proteins were then loaded onto Prometheus NT.48 nanoDSF-grade standard capillaries (PR-C002, NanoTemper Technologies), and the fluorescence signal was recorded under a temperature gradient ranging from 25 to 90 $^{\circ}$ C (incremental steps of 0.5 $^{\circ}$ C min⁻¹). The melting temperature (T_m) was calculated as the mid-point temperature of the melting curve using the PR ThermControl software 2.3.1 (NanoTemper Technologies, Munich, Germany). The melting curve was drawn using the GraphPad Prism 7.0 software; the values of the first derivative of 350/330 nm were displayed on the Y axis.

2.4. Enzyme Activity Assays

The experiment was performed as mentioned before in [22].

A fluorescent substrate harboring the cleavage site (indicated by $\downarrow$) of the SARS-CoV-2 M^{Pro} (Dabcyl-KTSAVLQ $\downarrow$ SGFRKM-E(Edans)-NH₂; Biosyntan, Berlin, Germany) and a buffer composed of 20 mM HEPES, 120 mM NaCl, 0.4 mM EDTA, 4 mM DTT, 20% glycerol, and 1% DMSO, pH 7.0, was used for the inhibition assay. In the fluorescence resonance energy transfer (FRET)-based cleavage assay, the fluorescence signal of the Edans generated due to the cleavage of the substrate by the M^{Pro} was monitored, using an Flx800 fluorescence spectrophotometer (BioTek, Santa Clara, CA, USA), at an emission wavelength of 460 nm with excitation at 360 nm. Initially, 10 μ L of the SARS-CoV-2 M^{Pro} WT at a final concentration of 50 nM, H172Y at 400 nM, E166V at 500 nM, Q189K at 300 nM, and S144A at 100 nM were pipetted into a 96-well plate containing 60 μ L of pre-pipetted reaction buffer. Then, 30 μ L of the substrate at varying concentrations (10, 20, 40, 80, 120, 160, 240, 320 μ M), dissolved in the reaction buffer, was added to reach a final volume of 100 μ L. The measurements were taken over 45 min.

A calibration curve was generated by the measurement of varied concentrations (from 0.04 to 6250 nM) of free Edans, with a gain of 80 in a final volume of 100 μ L reaction buffer. Initial velocities were determined from the linear section of the curve, and the corresponding relative fluorescence units per unit of time (Δ RFU/s) was converted to the amount of cleaved substrate per unit of time (μ M/s) by fitting to the calibration curve of free Edans.

Inner-filter effect corrections were applied for the kinetic measurements according to Liu et al. [23].

As saturation could be achieved, kinetic constants ($V_{\max}$ and K_m) were derived by fitting the corrected initial velocity to the Michaelis–Menten equation, $V = V_{\max} \times [S]/(K_m + [S])$, using the GraphPad Prism 7.0 software. k_{cat}/K_m was calculated according to the equation, $k_{\text{cat}}/K_m = V_{\max}/([E] \times K_m)$. Triplicate experiments were performed for each data point, and the value was presented as the mean $\pm$ standard deviation (SD).

2.5. Inhibitory Activity Determination of WT-M^{Pro} and M^{Pro} Mutants in Complex with 13b-K and Nirmatrelvir

Inhibition assays of the SARS-CoV-2 M^{Pro} against 13b-K or nirmatrelvir were performed using different inhibitor concentrations from 0 to 100 μM (0, 0.000063, 0.00019, 0.00056, 0.0017, 0.005, 0.015, 0.045, 0.1, 0.4, 1.2, 3.7, 11, 33, 100). The measurement time was 15 min.

First, 10 μL of enzyme (50 nM final enzyme concentration for WT, 400 nM for H172Y, 500 nM for E166V, 300 nM for Q189K, and 100 nM for S144A) was initially pipetted into a 96-well plate containing 59 μL of the reaction buffer (20 mM HEPES pH 7.0, 120 mM NaCl, 0.4 mM EDTA, 20% glycerol, 4 mM DTT). Subsequently, the corresponding concentration of the compound was added in drops with the M^{Pro} and reaction buffer, and the inhibitor–M^{Pro} mixture was incubated at 37 °C for 10 min.

Finally, the reaction was initiated by adding 30 μL of the FRET substrate (Dabcyl-KTSAVLQ↓SGFRKM-E(Edans)-NH₂; Biosyntan, Berlin, Germany) dissolved in the reaction buffer. The reaction was monitored at 37 °C for 45 min using a spectrofluorometer, namely the Tecan Spark (Tecan Group Ltd., Männedorf, Switzerland), at an emission/excitation wavelength of 460/360 nm.

Measurements of enzymatic activity were performed in triplicate and the value was presented as the mean $\pm$ standard deviation (SD). The initial velocity of the enzymatic reaction with the compounds was adjusted using inner-filter effect corrections and calculated by linear regression for the first minute of the progression curve. Inhibition rates were obtained as $(1 - (V_{\max}/V_0)) \times 100\%$. Inhibition constants K_i were calculated using the Morrison equation.

2.6. Analytical Ultracentrifugation

Sedimentation velocity runs were carried out in analytical ultracentrifuges, i.e., Optima AUC or ProteomeLab XL-I (Beckman Coulter, Brea, CA, USA), using An-50 Ti rotors at 50,000 rpm and 20 °C. Concentration profiles were measured with the absorption scanning optics at 230 nm using 3 or 12 mm standard double-sector centerpieces filled with 100 μL or 400 μL sample, respectively. Experiments were performed in a buffer containing 0.12 M NaCl, 20 mM HEPES pH 7.0, 10% (*v/v*) glycerol, 0.2 mM EDTA, and 1 mM TCEP in a concentration range of 0.25 to 10 μM SARS-CoV-2 M^{Pro}. After dilution, samples were allowed to equilibrate for at least 4 h at 20 °C prior to sedimentation analysis. To investigate the influence of nirmatrelvir or 13b-K on the monomer–dimer equilibrium of SARS-CoV-2 M^{Pro} mutants, proteins were diluted in the presence of 12 μM of the respective inhibitor.

For data analysis, a model for diffusion-deconvoluted differential sedimentation coefficient distributions (continuous *c(s)* distributions) implemented in the program SEDFIT [24] was used. The buffer density and viscosity and partial specific volumes were calculated from the buffer and amino acid compositions, respectively, with the program SEDNTERP [25] and were used to correct the experimental *s*-values to *s*_{20,w}. Signal-weighted average *s*-values, *s*_w, were obtained by integration of the *c(s)* distributions in the *s*-value range where monomers and dimers were observed using the program GUSI [26]. Monomer–dimer equilibrium dissociation constants, *K_d*, were determined by

fitting a monomer–dimer self-association model to the s_w isotherms using the program SEDPHAT [27]. As the dissociation of the H172Y mutant was incomplete, the sedimentation coefficient of the monomer was set to the value obtained for the E166V mutant (2.7 S), as determined by the fitting of the s_w isotherm. Figures showing the $c(s)$ distributions were obtained with the program GUSLI.

Protein concentrations were determined spectrophotometrically using the absorption coefficients at 280 nm as calculated from the amino acid composition [28] and are given in monomers throughout the text.

2.7. Crystallization and Diffraction Data Collection of WT M^{Pro} Free Enzymes and in Complex with Nirmatrelvir and 13b-K

The purified SARS-CoV-2 M^{Pro} proteins were concentrated to 14 mg/mL. For the complexes with nirmatrelvir and 13b-K, the proteins were then incubated overnight with a 5-fold molar excess of inhibitor, and the mixture was incubated overnight at 4 °C.

After being centrifuged at 13,000 rpm to remove precipitates, the supernatants of the free enzyme and the protein-compound mixture were then subjected to co-crystallization screening with commercially available kits (PACT premier™ HT-96, Morpheus and LFS Screen from Molecular Dimensions, Rotherham, UK), using an Art Robbins Instruments robot and the vapor diffusion sitting drop method at 20 °C.

Diffraction data from the free enzymes H172Y, E166V, Q189K, and S144A-M^{Pro} were collected from crystals grown under the conditions of LSF No.-F7 (0.1 M bis-tris propane pH 6.5, 0.2 M sodium acetate trihydrate, 20% w/v PEG 3350, 10% v/v ethylene glycol) for H172Y, LFS No.-F4 (0.1 M bis-tris propane pH 6.5, 0.2 M potassium thiocyanate, 20% w/v PEG 3350, 10% v/v ethylene glycol) for E166V, PACT No.-F6 (0.2 M sodium formate, 0.1 M bis-tris propane pH 6.5, 20% w/v PEG 3350) for Q189K, and LFS No.-F10 (0.1M bis-tris propane pH 6.50, 0.2 M sodium potassium phosphate pH 7.5, 20% w/v PEG 3350, 10% v/v ethylene glycol) for S144A.

Diffraction data from the H172Y, E166V, Q189K, and S144A M^{Pro}s in complex with 13b-K were collected from crystals grown under the conditions of LSF No.-H11 (0.1M bis-tris propane pH 8.5, 0.2 M potassium citrate tribasic monohydrate, 20% w/v PEG 3350, 10% v/v ethylene glycol) for H172Y, PACT No.-D8 (0.2M ammonium chloride, 0.1M tris pH 8.0, 20% w/v PEG 6000) for E166V, PACT No.-C5 (0.1M PCTP, pH 8.0, 25% w/v PEG 1500) for Q189K, and Morpheus No.-E5 (0.1M sodium HEPES, MOPS pH 7.5, 0.12 M ethylene glycol, 40% v/v PEG 500 MME, 20% w/v PEG 20000) for S144A.

Diffraction data from the H172Y, E166V, Q189K, and S144A M^{Pro}s in complex with nirmatrelvir were collected from crystals grown under the conditions of PACT No.-D5 (0.1M MMT 8.025% w/v PEG 1500) for H172Y and PACT No.-E11 (0.2 M sodium citrate tribasic dihydrate, 20% w/v PEG 3350) for Q189K. Crystals were then cryo-protected using 20% glycerol.

Fished crystals were flash-cooled using liquid nitrogen. Diffraction data were collected at 100 K at DESY PETRA III beamline P11 (Hamburg, Germany) using a Pilatus 6M detector (Dectris) and with synchrotron radiation at a wavelength of 1.0332 Å.

2.8. Diffraction Data Processing, Structure Solution, and Refinement

The programs XDSapp [29], Pointless [30,31], and Scala [30,31] were used for dataset processing, with Molrep [31,32] to solve the phase problem via the molecular replacement method using the SARS-CoV-2 M^{Pro} free enzyme (PDB code: 6Y2E), SARS-CoV-2 M^{Pro} in complex with nirmatrelvir (PDB code: 7vh8), and SARS-CoV-2 M^{Pro} in complex with 13b-K (PDB code: 6Y2G) as search models. All compounds were built into Fo-Fc difference densities using the Coot software 0.9.8.91 [33] and structures were refined using the Ref-

mac5 program [34]. All graphical illustrations and RMSD values were calculated using PyMOL 4.6 [35].

Diffraction data and model refinement statistics are displayed in Tables S1 and S2.

3. Results

The crystal structures of the H172Y-, E166V-, S144A-, and Q189K-SARS-CoV-2 M^{Pro} free enzyme mutants and in complex with 13b-K were determined at resolutions ranging from 1.69 to 2.20 Å. In addition, the crystal structures of the H172Y- and Q189K-SARS-CoV-2 M^{Pro} in complex with nirmatrelvir (PF-07321332) were also determined at 2.7 and 1.68 Å resolutions, respectively (Tables S2 and S3) [36–38].

In total, we determined 10 crystal structures of M^{Pro} mutants as free enzymes or in complex with 13b-K or nirmatrelvir. The crystal structures displayed one of four space groups (see Tables S2 and S3), with either two M^{Pro} protomers (one dimer) in the asymmetric unit of the crystal (space groups $P2_12_12_1$ or $P2_1$) or one M^{Pro} protomer (half a dimer) in the asymmetric unit (space groups $C2$ or $P2_12_12$).

The crystal structures of the wild-type (WT, Wuhan-Hu-1 strain (NC_045512.2)) M^{Pro} free enzyme [20] (PDB: 6Y2E), the WT M^{Pro} in complex with 13b-K (PDB: 6Y2G), and the WT M^{Pro} in complex with nirmatrelvir [14] (PDB: 7VH8) were used for structural comparisons.

We also used sedimentation velocity analysis by analytical ultracentrifugation (AUC) in order to investigate the influence of the four naturally occurring point mutations on the dimer stability of the SARS-CoV-2 M^{Pro} and the influence of 13b-K and nirmatrelvir on dimerization.

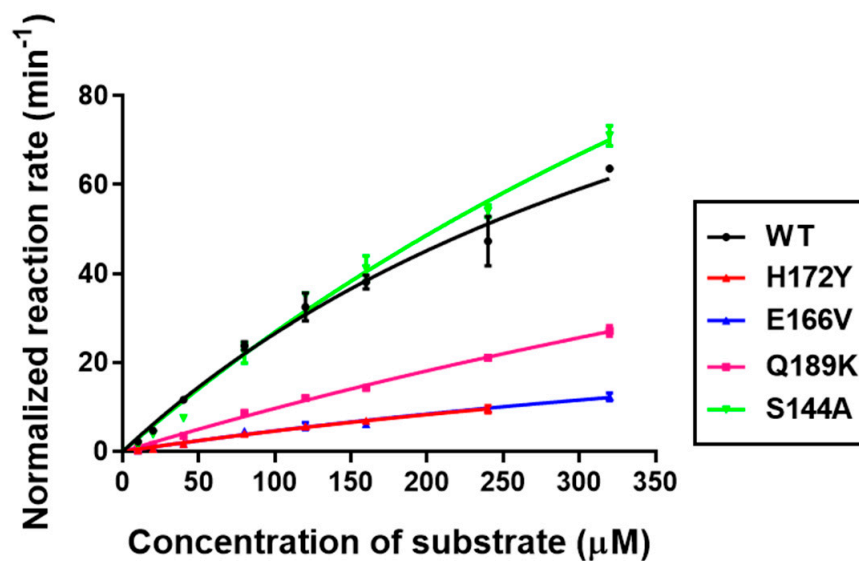
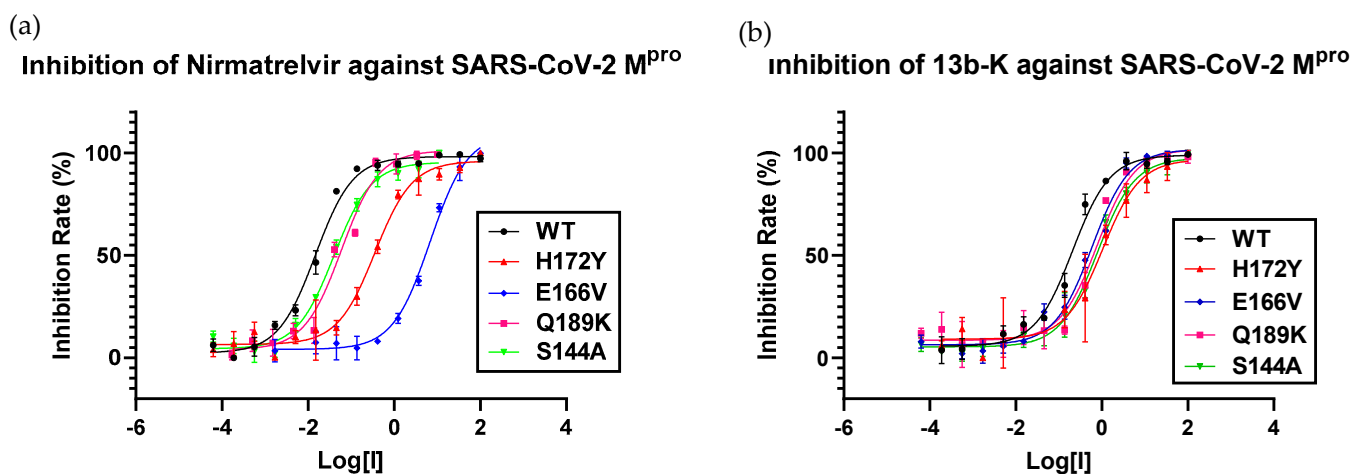
In the presence of a 1 mM concentration of the reductant tris (2-carboxyethyl) phosphine (TCEP), the WT M^{Pro} forms stable dimers, which only start to slightly dissociate at a concentration of 0.25 µM (Figure S1a). Unfortunately, lower M^{Pro} concentrations are not accessible with the absorbance optics of the AUC. Therefore, the dissociation constant (K_d) has to be significantly lower than 0.25 µM. In an earlier study, we found that, in the absence of a reductant, the M^{Pro} dimer was much less stable, with a K_d of about 2.5 µM [1]. This significant difference in dimer stability is due to the redox regulation of the M^{Pro} involving a disulfide bond between the catalytic cysteine and a proximal cysteine, as well as an allosteric SONOS bridge [39]. Since we were interested in the oligomerization state of the active enzyme, all experiments in this study were performed under reducing conditions.

3.1. Activity of Mutants, Stability of Proteins, and Influence of Inhibitors on Their Activity

The H172Y mutant was first characterized. Its thermal stability was evaluated using nano-differential scanning fluorimetry (nanoDSF) to determine the melting temperature (T_m) of the protein. A marked decrease in T_m was observed (see Table 1 and Figure S2), indicating the compromised stability of the M^{Pro} due to this mutation. More than a 4 °C decrease in the T_m value for the mutant free enzyme was observed, in comparison to the WT M^{Pro} free enzyme (57.5 °C for WT_free vs. 53.4 °C for H172Y_free). Even more pronounced reductions in thermal stability were observed in inhibitor-bound forms, with drastic decreases (10–15 °C) in the T_m values for the H172Y–nirmatrelvir and H172Y–13b-K complexes compared to their WT counterparts (74.0 °C and 67.5 °C for WT vs. 58.5 °C and 57.7 °C for H172Y, respectively). In addition to reduced stability, the catalytic efficiency and inhibitor sensitivity of the H172Y mutant were also found to be reduced. Only ~16% of the enzymatic activity of the WT M^{Pro} remained ($k_{cat}/K_m = 5355.26 \pm 1447.83 \text{ M}^{-1} \cdot \text{s}^{-1}$ for WT vs. $k_{cat}/K_m = 863.34 \pm 380.32 \text{ M}^{-1} \cdot \text{s}^{-1}$ for H172Y; see Table 1 and Figure 1. Based on the IC₅₀ values, the H172Y mutant displayed increased resistance to both 13b-K and nirmatrelvir, with resistance factors of 4.7 and 23.4, respectively (see Table 1 and Figure 2).

Table 1. Summary of the kinetic constants, IC₅₀ values, resistance factors, and melting temperatures of the M^{Pro} WT, H172Y, E166V, Q189K, and S144A mutants.

	Enzyme Activity (k_{cat}/K_m , M ⁻¹ ·s ⁻¹)	IC ₅₀ (nM) ± SD		rR		K _i		Free Enzyme (°C)	T _m	
		Nirm. (nM)	13b-K (nM)	Nirm.	13b-K	Nirm. (nM)	13b-K (nM)		Nirm. (°C)	13b-K (°C)
WT	5355.26 ± 1447.83	15 ± 2	210 ± 47	1	1	0.86	~4	57.5	74.0	67.5
H172Y	863.34 ± 380.32/16.12%	344 ± 88	997 ± 468	23.4	4.7	1211	1669	53.4	58.5	57.7
E166V	860.28 ± 361.93/16.06%	6030 ± 1911	595 ± 154	410.0	2.8	28,575	29,446	55.3	56.8	59.4
Q189K	1736.16 ± 533.05/32.42%	60 ± 21	790 ± 208	4.1	3.8	6115	7160	57.4	73.1	68.3
S144A	4983.46 ± 1700.84/93.05%	40 ± 9	910 ± 290	2.7	4.3	3598	4484	58.0	66.4	62.8

**Figure 1.** Reaction rate vs. substrate concentration using the FRET assay for 50 nM SARS-CoV-2 M^{Pro} WT, 400 nM H172Y, 500 nM E166V, 300 nM Q189K, and 100 nM S144A.**Figure 2.** Inhibition rates of nirmatrelvir and 13b-K against SARS-CoV-2 M^{Pro} WT, H172Y, E166V, Q189K, and S144A. Inhibition rate vs. Log[I], where [I] is the concentration of the inhibitor. (a) Illustrate the Inhibition of Nirmatrelvir against M^{Pro} and (b) Illustrate the Inhibition of 13b-K against M^{Pro}.

After the substitution of Glu166 with Val, the enzyme activity decreased by more than 84% ($k_{cat}/K_m = 860.28 \pm 361.93 \text{ M}^{-1} \cdot \text{s}^{-1}$). Compared to the WT, the E166V mutant is 2.8-fold more resistant to 13b-K and exhibits exceptionally high 410.0-fold resistance

to nirmatrelvir (see Table 1 and Figures 1 and 2). The stability of the enzyme in complex with nirmatrelvir is more affected by the E166V mutation ($\sim 17^\circ\text{C}$ decrease in the melting temperature, T_m) compared to the complex with 13b-K ($\sim 8^\circ\text{C}$ decrease in the T_m value). However, only a slight reduction in the T_m value is observed for the free E166V enzyme ($\sim 2^\circ\text{C}$) (see Table 1 and Figure S2, in Supplementary Materials).

For the Q189K mutant, a decrease in the enzymatic activity of the free enzyme is observed (more than 67% of the enzymatic activity is lost ($k_{cat}/K_m = 1736.16 \pm 533.05 \text{ M}^{-1}\cdot\text{s}^{-1}$)). However, the stability of the M^{Pro} is not affected (T_m values of Q189K are comparable to the WT values; see Table 1).

Confirming Pfizer's report for healthcare providers [8] (FDA, 2021), S144A has slightly reduced enzymatic activity ($k_{cat}/K_m = 4983.46 \pm 1700.84 \text{ M}^{-1}\cdot\text{s}^{-1}$) compared to the WT ($k_{cat}/K_m = 5355.26 \pm 1447.83 \text{ M}^{-1}\cdot\text{s}^{-1}$). About 93% of the wild-type activity is preserved (Table 1 and Figure 2). Based on the T_m values of the S144A mutant (58°C for the free enzyme, 62.8°C for the complex with 13b-K, and 66.4°C for the complex with nirmatrelvir), we conclude that the stability of the S144A free enzyme is not affected compared to the WT free enzyme (57.5°C). However, the S144A complexes with 13b-K and nirmatrelvir show significant decreases in their T_m values (67.5°C for the WT in complex with 13b-K and 74°C for the WT in complex with nirmatrelvir) (Table 1 and Figure S2).

3.2. Stability of Protein Dimers in Sedimentation Velocity Experiments

AUC experiments reveal the lower stability of the H172Y M^{Pro} dimer compared to the WT M^{Pro} (see Figure 3c and Figure S1b,d). The H172Y mutant dissociates partially in the concentration range investigated. K_d values below $0.25 \mu\text{M}$ for the WT and of about $0.3 \mu\text{M}$ for H172Y were estimated from the s_w isotherms. For the WT M^{Pro} and the H172Y M^{Pro} mutant, we address the question of whether the 13b-K and nirmatrelvir inhibitors influence the stability of the dimer. A concentration of $12 \mu\text{M}$ of either inhibitor has a significant stabilizing effect on both protein complexes (Figure S1c,d and Figure 4a–d). Both the WT and the H172Y mutant no longer show any tendency to dissociate, even at a concentration of $0.25 \mu\text{M}$. A possible difference in the stabilizing effects of 13b-K and nirmatrelvir on the dimer stability of the WT or the H172Y mutant could not be determined, since neither protein showed any dissociation in the presence of either inhibitor in the concentration range studied.

The sedimentation velocity analyses show that the dimer of E166V M^{Pro} is even less stable than that of H172Y (see Figure 3c,d and Figure S1b,e). The M^{Pro} E166V dimer dissociates significantly even in the presence of 1 mM TCEP. In contrast to the dissociation observed for the WT in the absence of a reductant, in the case of E166V and H172Y, the reaction seems to be fast relative to the time scale of sedimentation in the presence of TCEP. This can be seen from the fact that, in the $c(s)$ distributions, the peak position gradually increases with the protein concentration [40]. For the E166V dimer, a K_d value of about $3 \mu\text{M}$ can be determined from the fit of the s_w isotherm. A concentration of $12 \mu\text{M}$ of nirmatrelvir or 13b-K has a stabilizing effect on the E166V mutant (Figure S1e). Interestingly, 13b-K stabilizes the E166V dimer even more strongly than nirmatrelvir (see Figure S1e and Figure 4e,f).

Confirming the stability study, the sedimentation velocity analysis reveals that Q189K forms stable dimers in the presence of 1 mM TCEP, even without an inhibitor, showing that the Q189K mutation does not significantly influence the monomer–dimer equilibrium (Figure 3a). The S144A mutation slightly destabilizes the M^{Pro} dimer. However, this effect is less pronounced than that seen with the H172Y mutant (Figure S1b and Figure 3b).

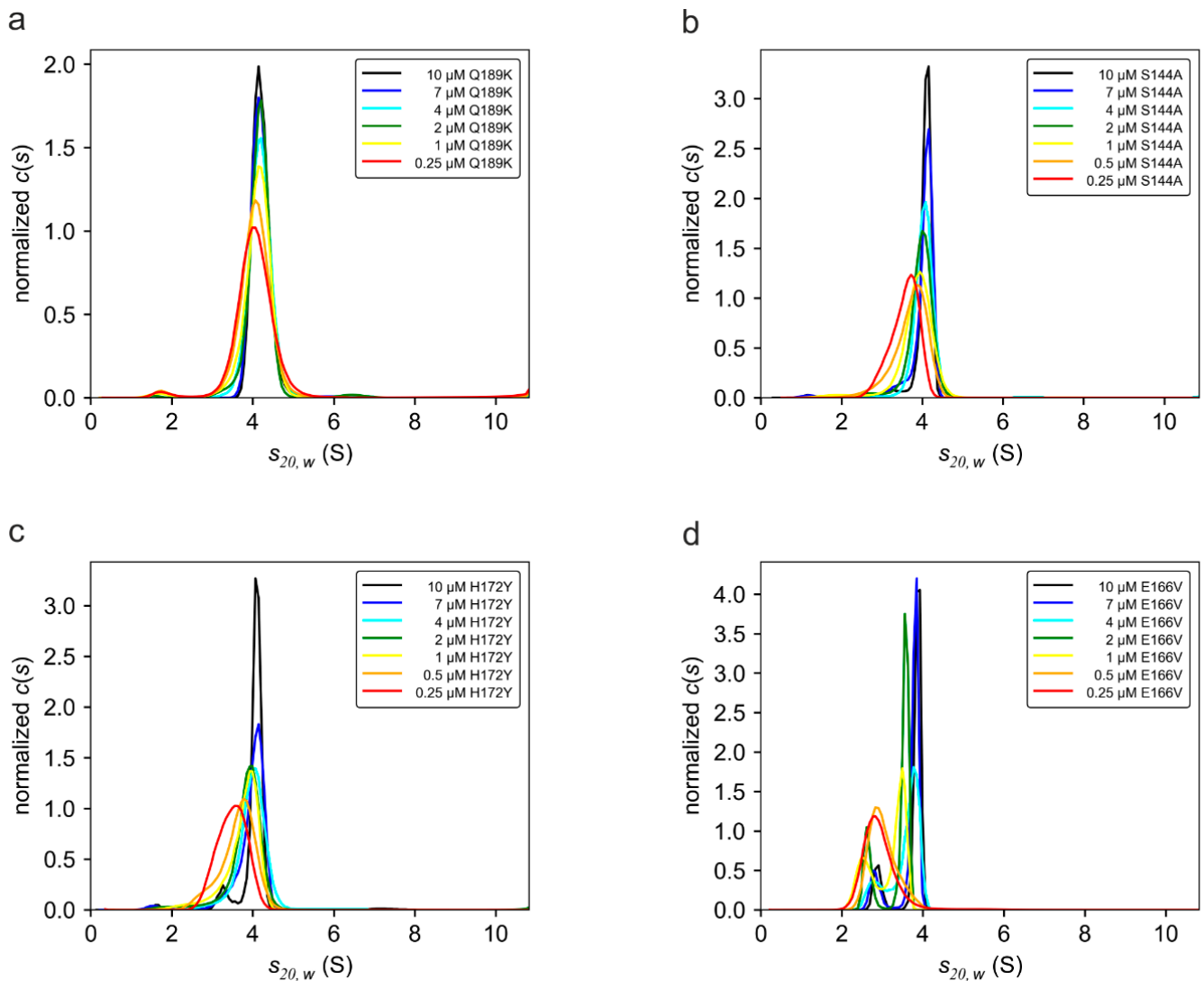


Figure 3. Naturally occurring point mutations affect the dimer stability of the SARS-CoV-2 M^{Pro}. Mutants of the SARS-CoV-2 M^{Pro} were analyzed by sedimentation velocity analysis in an analytical centrifuge under reducing conditions. Sedimentation coefficient distributions $c(s)$ in a concentration range of 0.25 to 10 μM M^{Pro} were obtained for mutants Q189K (a), S144A (b), H172Y (c), and E166V (d). Whereas the Q189K mutation has no influence on the monomer–dimer equilibrium of the SARS-CoV-2 M^{Pro} (compare to Figure S1a), the other mutants show increased dissociation at decreasing protein concentrations. From the extent of dissociation, the stability of the dimers can be ranked as WT = Q189K > S144A > H172Y > E166V. As the peak positions shift with decreasing protein concentrations, the dissociation seems to be fast relative to the time scale of sedimentation [40]. For better visibility, $c(s)$ distributions were normalized to the same peak areas.

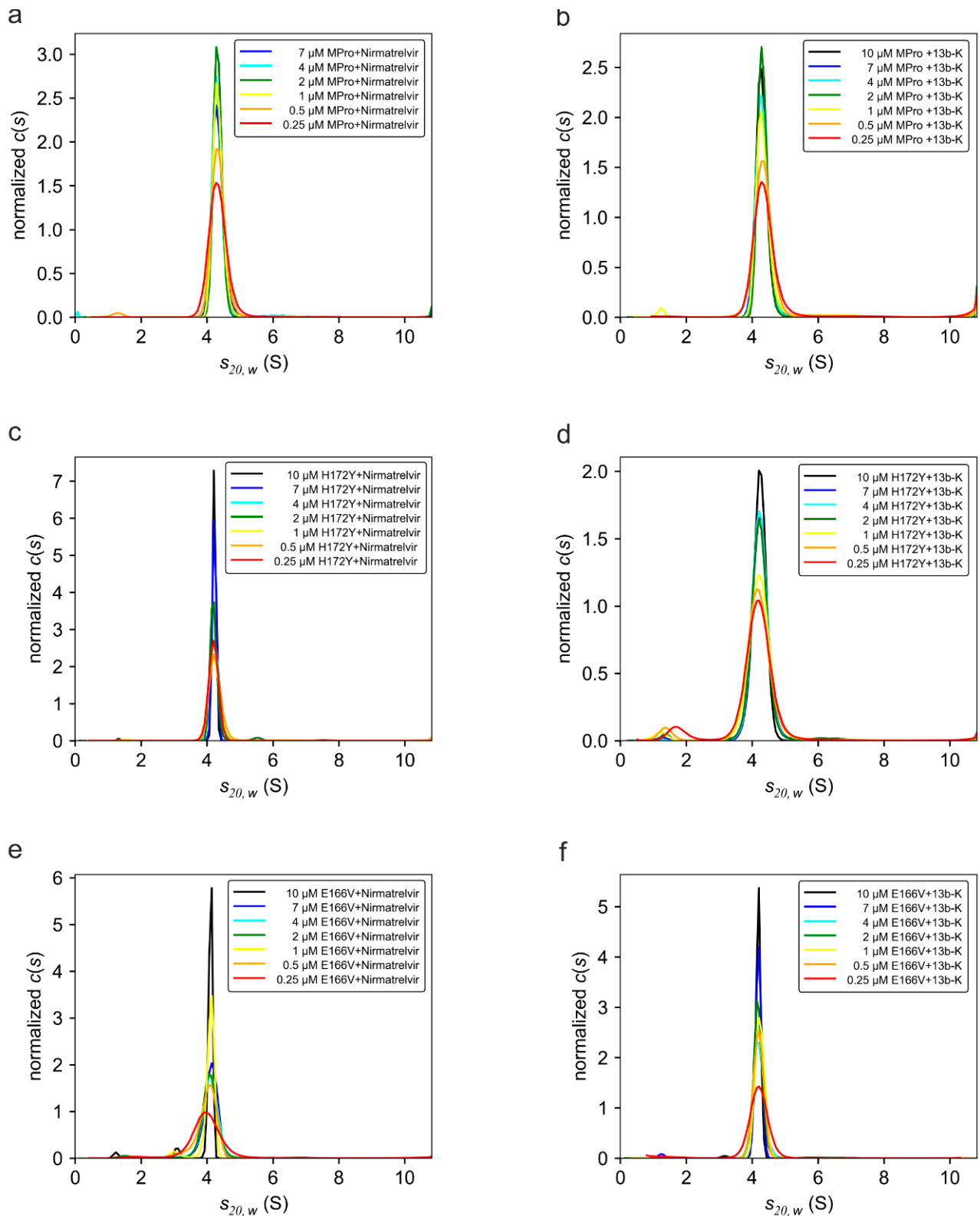


Figure 4. Inhibitors nirmatrelvir and 13b-K stabilize SARS-CoV-2 M^{Pro} dimers. Sedimentation coefficient distributions of WT SARS-CoV-2 M^{Pro} (a,b), H172Y (c,d), and E166V (e,f) in a concentration range of 0.25 to 10 μ M in the presence of 12 μ M nirmatrelvir (a,c,e) or 13b-K (b,d,f), respectively. Both inhibitors stabilize the dimeric forms of all three proteins. In the case of E166V, the stabilizing effect of 13b-K is even more pronounced. For better visibility, c(s) distributions were normalized to the same peak areas.

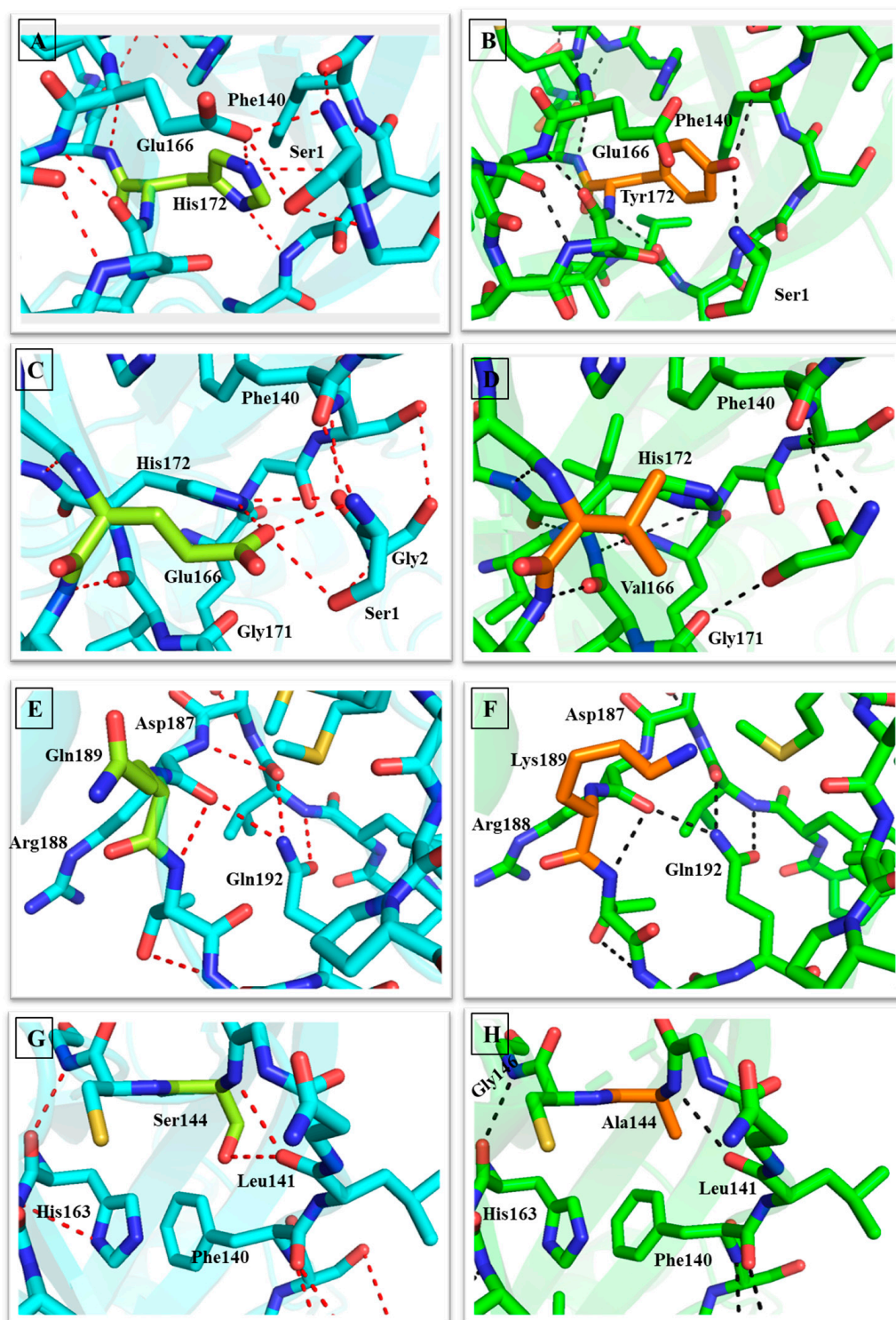


Figure 6. Structural interactions of the N-finger (Ser1) at the dimer interface of the MP^{Pro} WT free enzyme ((A,C,E,G); PDB: 6Y2E), H172Y free enzyme ((B); PDB: 8CA8), E166V free enzyme ((D); PDB: 8C9P), Q189K free enzyme ((F); PDB: 8CA6), and S144A free enzyme ((H); PDB: 8C9L). Mutated amino acids are colored in orange, and the corresponding amino acids in the WT are colored in light green. Red and black dashes highlight H-bonds.

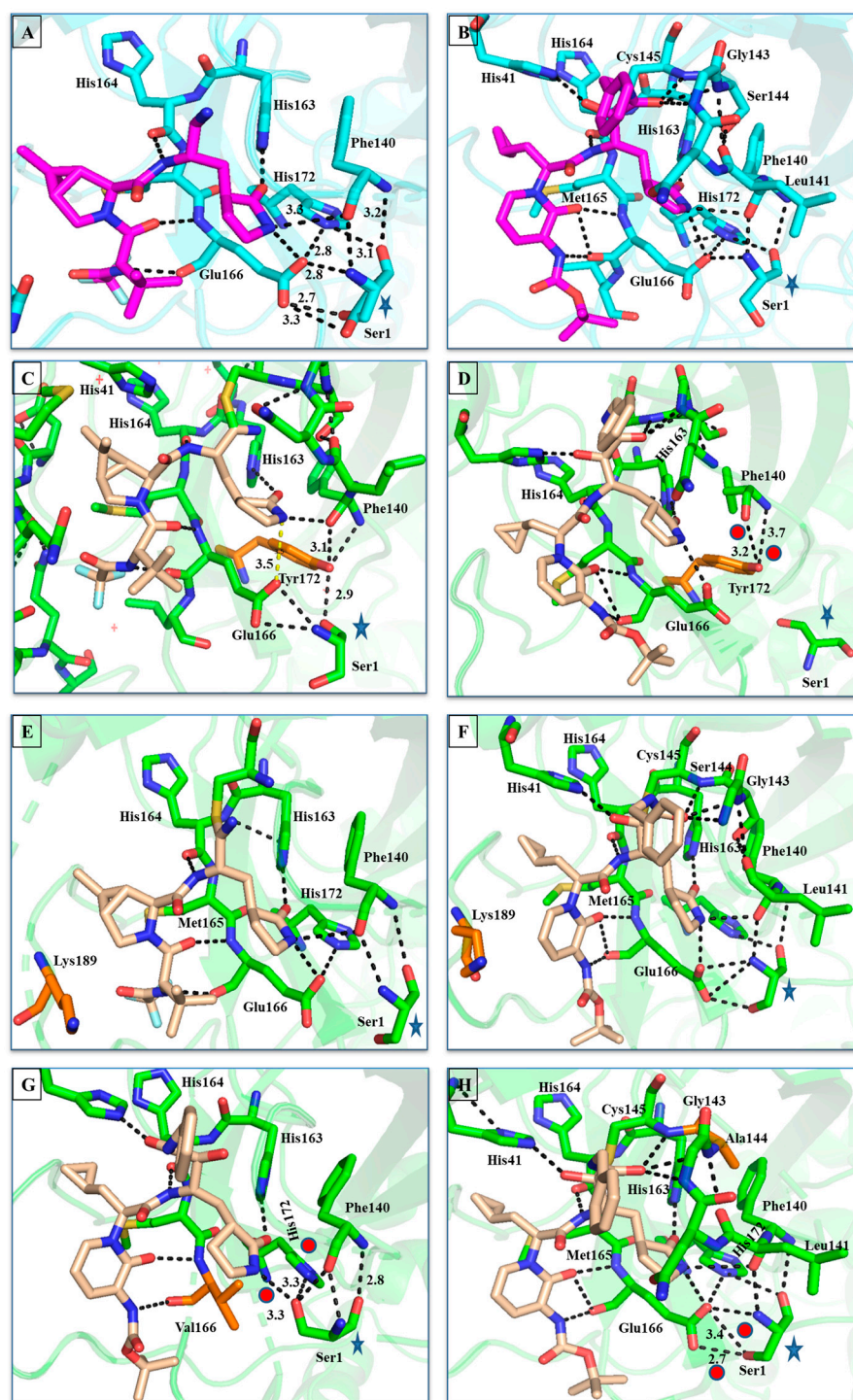


Figure 7. Crystal structures of M^{Pro} WT in complex with nirmatrelvir ((A); PDB: 7VH8), M^{Pro} WT in complex with 13b-K ((B); PDB: 6Y2G), H172Y in complex with nirmatrelvir ((C); PDB: 8CAE), H172Y in complex with 13b-K ((D); PDB: 8CAC), Q189K in complex with nirmatrelvir ((E); PDB: 8C9U), Q189K in complex with 13b-K ((F); PDB: 8C9Q), E166V in complex with 13b-K ((G); PDB: 8CAJ), and S144A in complex with 13b-K ((H); PDB: 8C90). Nirmatrelvir and 13b-K are highlighted in magenta for the WT and in tint for the mutant. The mutated amino acid residues are highlighted in orange. Black dashes highlight H-bonds, yellow dashes highlight other interactions (not H-bonds), and stars highlight Ser1* from the adjacent molecule.

3.3.2. E166V-SARS-CoV-2 M^{Pro}

In contrast to H172Y, no relevant backbone displacement of the N-finger is found for the E166V mutation. However, an examination of the E166V-SARS-CoV-2 M^{Pro} structure in

complex with 13b-K shows the displacement of the backbone of a series of residues from Glu46 to His80 (r.m.s. displacement of about 0.8 Å compared to the WT), from Asn214 to Thr224 (0.5 Å), and from Gln273 to Thr280 (0.6 Å). All these residues are situated at the protein surface (Figure 6C,D).

The strong binding of inhibitors in the S1 binding side of the M^{Pro} enzyme requires a series of interactions between the P1 γ -lactam of the inhibitor and four different amino acid residues (His163, Glu166, His 172, and Ser1*).

A series of H-bonds (between Glu166 O ϵ 2 and Ser1* N, 2.6 Å; between His172 N ϵ 2 and Glu166 O ϵ 2, 2.9 Å; between Phe140 O and the nitrogen of the P1-lactam moiety, 3.0 Å; and between Glu166 O ϵ 2 and the nitrogen of the P1-lactam moiety, 3.1 Å) are totally lost. This leads to an increased distance between the carbonyl oxygen of the P1 γ -lactam and the His163 N ϵ 2 (from ~2.6–2.8 to ~3.3 Å), possibly leading to the decreased binding affinity of the P1 moiety of the inhibitor.

However, two new contacts between Ser1* O γ and Gly170 O (2.9 Å) in the free enzyme and between the nitrogen of the P1 γ -lactam and Ser1* O γ (3.3 Å) in the complex with 13b-K are formed (Figure 7G).

Interestingly, after the nucleophilic attack on the 13b-K C α by the thiol of Cys145, the oxygen atom of the P1' benzyl group (originally pointing into the oxyanion hole in the SARS-CoV-2 M^{Pro} wild type in complex with 13b-K) points away from the oxyanion hole, resulting in the loss of two strong H-bonds to Ser144 N (2.9 Å) and Cys145 N (2.5 Å) and forming a new H-bond with His41 (2.6 Å). Due to the change in configuration of the chiral carbon bearing the hydroxyl group, from S to R, the oxygen atom of the P1 γ -lactam points into the oxyanion hole, losing the H-bond with His41 NE2 and forming three new H-bonds with Gly 143 N (3.3 Å), Ser 144 N (3.5 Å), and Cys145 N (3.1 Å).

3.3.3. Q189K-SARS-CoV-2 M^{Pro}

Upon superimposing the wild type with the mutant structures, overall RMSD values of 0.5 Å, 0.3 Å, and 0.4 Å for the free enzyme, the complex with nirmatrelvir, and the complex with 13b-K, respectively, are obtained (Figure 6E,F).

The Q189K mutation leads to the displacement of a number of residues from His41 to Ser81, from Met165 to Thr198, and from Phe220 to Ser301 in the free enzyme. However, no significant displacement for the enzyme in complex with 13b-K and nirmatrelvir is observed.

The displacement of the most conserved amino acid residues, Glu166 and His172 (with an RMSD value of 0.8 Å), explains the drastic decrease in the enzymatic activity of the free Q189K enzyme.

Moreover, the H-bond between Glu166 O ϵ 1/O ϵ 2 and H172 N ϵ 2 (~2.9 Å) is lost in Q189K in complex with 13b-K, but not in the free Q189K enzyme or in complex with nirmatrelvir. No new interactions created by the newly introduced Lys189 were observed (Figure 7E,F).

3.3.4. S144A-SARS-CoV-2 M^{Pro}

The polypeptide regions most affected by the backbone displacement in the free enzyme comprise Ser1–Arg4 (from the N-finger), Met165–Val171, Gln192–Asp197, and Arg298 to the C-terminus (Figure 6G,H).

An examination of the S144A M^{Pro} structure in complex with 13b-K shows that chain B is more affected by the displacement compared to chain A. The displacement of the backbone of a series of residues from Ile59 to Pro96 (~0.3 Å RMSD compared to the WT), from Ile213 to Phe230 (~0.4 Å RMSD), and from Gly251 to Thr280 (~0.4 Å RMSD) in chain B is observed. Interestingly, all these displaced residues are situated at the protein surface.

Due to the mutation, the weak interaction between Ser144 O γ and the oxygen of the P1' moiety (~ 3.7 Å) is lost. Furthermore, the H-bond between Ser144 O γ and Leu141 O (~ 2.6 – 2.7 Å) in the free enzyme and the complex with 13b-K is lost, explaining the small decrease in the enzymatic activity (Figure 7H).

4. Discussion

We characterized the nirmatrelvir-resistant M^{Pro} mutants H172Y, E166V, Q189K, and S144A in terms of enzyme kinetics and by biophysical as well as crystallographic methods. In addition, we tested our own M^{Pro} inhibitor 13b-K against these mutant M^{Pro} enzymes. Sedimentation velocity characterization of the M^{Pro} WT and all four mutants was carried out using analytical ultracentrifugation (AUC).

The AUC results show that, while the Q189K mutation does not affect the stability of the M^{Pro} dimer, increasing dissociation is observed for the S144A, H172Y, and E166V mutants (see Figure 3 and Figure S1). From the obtained sedimentation coefficient distributions, signal-weighted average *s*-values (*s_w*) have been determined at each protein concentration. For the least stable mutants, H172Y and E166V, the resulting binding isotherms were used to fit a monomer–dimer self-association model to the data and yielded *K_d* values of 0.3 μ M and 3 μ M, respectively (Figure S1b).

According to the nanoDSF results, 13b-K stabilizes the E166V even more strongly than nirmatrelvir (see Figure S2). The melting curves show that the stability of the M^{Pro} dimer decreases in the following order: WT = Q189K > S144A > H172Y > E166V.

Consistent with previously reported results (Lewandowski and Tan) [41], our data demonstrate that the free enzyme S144A exhibits comparable enzyme activity to the free WT enzyme.

One might suppose that the structural difference between histidine and tyrosine is not large, but, in fact, the structural consequences of the H172Y mutation are significant. The hydroxyl group of Tyr172 introduces a new, relatively weak hydrogen-bonding interaction with the backbone amide of Phe140 (with a distance of 3.0 Å; Figure 4c). Clayton et al.'s MD simulation data suggested significant disruption in the S1 pocket interactions with the N-terminus due to the mutation [22]. Our X-ray structures of the H172Y free enzyme and H172Y in complex with nirmatrelvir confirm this suggestion.

The M^{Pro}'s thermal stability is strongly reduced by the H172Y and E166V mutations, even upon inhibitor binding. The melting temperatures of the enzyme–inhibitor complexes decrease significantly compared to the WT. Four H-bonds are lost for both mutants, and the resistance against nirmatrelvir is very high compared to the WT, Q189K, and S144A enzyme–inhibitor complexes (*IC*₅₀ values about 23.4-fold for H172Y and 410.0-fold for E166V in complex with nirmatrelvir, compared to the WT enzyme–nirmatrelvir complex). Glu166 forms three H-bonds with nirmatrelvir and four H-bonds with 13b-K. The E166V mutation eliminates the interactions with the gamma-lactam moiety, which is crucial for inhibitor binding [42]. We determined also the structure of E166V in complex with nirmatrelvir (data not shown); however, the occupancy of the inhibitor is too low (about 0.5), confirming the high resistance of the SARS-CoV-2 E166V mutant against nirmatrelvir and the low potency of the inhibitor against the mutant. The higher *T_m* value of the complex, compared to the free enzyme, confirms the relative binding of nirmatrelvir to the enzyme, albeit with low occupancy.

Our own inhibitor, 13b-K, shows cross-resistance with nirmatrelvir against all four mutations, with *IC*₅₀ values increased 2.8–4.7-fold. However, S144A's resistance is less pronounced against nirmatrelvir than 13b-K, whereas H172Y, E166V, and Q189K show greater resistance to nirmatrelvir than to 13b-K.

The structural and biochemical analysis indicates that H172Y and E166V display severe disturbances of the M^{Pro} homodimer formation and therefore highly reduced catalytic activity. In contrast, Q189K and S144A display relatively undisturbed three-dimensional structures and retain much of their catalytic activity while showing significant nirmatrelvir resistance. Accordingly, it can be assumed that these two mutations pose a higher threat should they spread in the population.

In order to overcome drug resistance and reduce the duration of treatment, Li et al. [2] suggested the development of dual- or triple-drug combinations for COVID-19 treatment. This treatment is a combination of a protease inhibitor and a polymerase inhibitor with a pharmacokinetic booster. Previous studies on HIV and HCV treatments based on multiple-drug combinations were very successful in preventing the development of resistance [43,44].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cryst15090758/s1>. 1. Figure S1: Dimer stability and the influence of nirmatrelvir and 13b-K on dimerization; 2. Table S1: Summary of the important existing, lost and newly formed contacts and hydrogen bonds due to the mutations; 3. Figure S2: Melting curves; 4. Table S2: Diffraction data and model refinement statistics of H172Y and E166V mutants; 5. Table S3: Diffraction data and model refinement statistics of H172Y and E166V mutants.

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