Dear Editor,

The ongoing SARS-CoV-2 pandemic continues to be a significant threat to global health. First reported in November 2021, the Omicron variant (B.1.1.529) is more transmissible and can evade immunity better than previous SARS-CoV-2 variants, fueling an unprecedented surge in cases. To produce functional proteins from its polyprotein, SARS-CoV-2 relies on the cysteine proteases Nsp3/Nsp5 and Plpro, which are the most promising SARS-CoV-2 antivirals. On December 22, 2021, the Food and Drug Administration (FDA) issued an Emergency Use Authorization (EUA) for PAXLOVID, a ritonavir-boosted formulation of nirmatrelvir and PF-00835231 (Fig. 1d). Interestingly, when these inhibitors are used in combination, their antiviral potency remains additive, and their interactions with the active site allow for more effective drug resistance. Moreover, the Thr198 sidechain rotates ~90°, placing its hydroxyl group in close distance to Glu240. As a result of these structural changes, OMpro has a nearly identical structure to WT Mpro (Fig. 1f).4 Overall, OMpro has a melting temperature of 56.2 ± 0.2 by 2.6 °C (Fig.1b). Despite its lower melting temperature (Tm) of 53.6 ± 0.1 °C; lower than the WT Mpro Tm of 57.2 ± 0.2 by 2.6 °C (Fig. 1b). In addition, the P132H mutation in the main protease of Omicron SARS-CoV-2 decreases thermal stability without compromising catalysis or small-molecule drug inhibition.

Using an established Förster resonance energy transfer (FRET) assay to monitor proteolytical activity,7 we show that OMpro and wild-type (WT) Mpro have equivalent affinity and catalytic constants for its substrate (Fig. 1a, b). However, subsequent melting temperature experiments reveal OMpro has a melting temperature (Tm) of 53.6 ± 0.1 °C; lower than the WT Mpro Tm of 56.2 ± 0.2 by 2.6 °C (Fig. 1b). Despite its lower melting temperature, OMpro and WT Mpro hydrolyze their substrates at comparable rates for up to 24 h at 37 °C (Fig. 1c). Further biochemical analysis suggests that OMpro and Mpro are equally susceptible to covalent inhibitors such as GC-376, PF-07321332 (nirmatrelvir), and PF-00835231 (Fig. 1d). Interestingly, when these inhibitors are incubated with WT Mpro or OMpro, their melting temperatures converge, despite the different melting temperature of their apo forms (Fig. 1e). This suggests that inhibitors may stabilize OMpro to a greater extent than WT Mpro. These biochemical/biophysical results are in line with the equivalent antiviral potency of these molecules against WT and Omicron and the lack of observable differences in the active site.5–8

In parallel to our study, Ullrich et al. also characterized six Mpro mutants identified from the circulating SARS-CoV-2 variants.5 The catalytic efficiency (kcat/Km) values for WT and Omicron Mpro were 0.016 and 0.023 s⁻¹ μM⁻¹, similar to the values obtained in our study (Fig. 1b). In addition, the Pfzer team reported that PF-07321332 (nirmatrelvir) retained a potent inhibitory constant (K) against Omicron P132H Mpro (K = 0.635 nM), similar to WT (K = 0.933 nM).10 Our independent study further confirmed these results. The crystal structure of OMpro in complex with GC-376 was determined at 2.05 Å resolution in the monoclinic space group I2 with a Rwork/Rfree of 0.179/0.219 (Supplementary information, Table S1; PDB ID 7TOB). The unit cell is the same as many previously solved WT Mpro crystal structures where a = 45.19 Å, b = 52.99 Å, c = 113.01 Å and α = 90.0°, β = 100.5°, γ = 90.0°. Overall, OMpro has a nearly identical structure to WT Mpro (Fig. 1f). The most pronounced differences involve the area around the mutation associated with OMpro, P132H. Found 22 Å from the catalytic cysteine Cys145, P132H lies between the catalytic domain and the dimerization domain, and thus, it does not impart any direct structural changes to the active site.

Clear electron density shows that His132 forms n-stacking interactions with the sidechain of Glu240 (Fig. 1g). As the imidazole sidechain of histidine is protonated near physiological pH, the interaction with Glu240 may be further strengthened through electrostatic interactions. Additionally, there is a newly formed water-mediated hydrogen bond with Glu240. We also find that Glu240 reorients itself toward the core to accommodate the bulkier sidechain, where it forms a new hydrogen bond with Thr198 (Fig. 1h). Consequently, the Thr198 sidechain rotates ~90°, placing its hydroxyl group in close distance to Glu240. As a result of these conformational changes, certain portions of the enzyme appear to move very slightly, ~0.5 Å (Fig. 1f). We speculate that the lower thermal stability of apo OMpro may be due to minor residue adjustments to accommodate the bulkier His sidechain, eventually destabilizing its structure. Because the mutation occurs at the interface between the dimerization domain and the catalytic domain, some of these movements can also affect intramolecular packing. Residue 132 is also located at the turn between two β-sheets, a position that naturally favors the cyclic sidechain of proline.

Although the P132H mutation does not appear to reduce enzymatic activity and inhibitor binding (Fig. 1a–d), the decrease in thermal stability (Fig. 1b) indicates that protein flexibility may be greater, which plays an important role in enzyme evolution, especially to broaden substrate profile or to alter ligand binding. Mpro can recognize a wide range of peptide substrates, although its P1 position preferentially binds to glutamine. Further studies on whether P132H and other mutations can influence enzymatic activity for larger substrate libraries or other known ligands will be necessary. In addition, the effect of the Mpro P132H mutant on the viral replication remains elusive, and further works need to generate recombinant viruses to study the viral replication kinetics and tropism.

Extensive sequencing of SARS-CoV-2 isolates has provided unprecedented insights into the mutations that occur in the viral RNA genome.6 Based on the annotations provided through CoVsurver enabled by GISAID (www.gisaid.org/epiflu-applications/covsurver-mutations-app), mutations of Nsp5 appear mostly...
stochastic (Supplementary information, Fig. S1); however, several positions have a disproportionately large number of mutations. Of the top 25 most common mutants (Fig. 1i), three are found on P132: P132H (489,444 occurrences; EPI_ISL_8931050), P132L (8813 occurrences; EPI_ISL_8768027), and P132S (7452 occurrences; EPI_ISL_8925342). L89F, K90R, and K88R are the second, third, and seventh, most common mutations, suggesting this β-sheet is also a hotspot. Notably, none of the 25 most common mutants involve residues in the active site or at the dimerization interface (Fig. 1j). With several exceptions, including P132H, the resulting amino acid is often similar in size and physicochemical properties, such as K → R. However, SARS-CoV-2 has not yet encountered Mpro antivirals. If nothing else, SARS-CoV-2 has taught us that widespread proliferation, low fidelity genome synthesis, and selective pressure might quickly produce drug-resistant phenotypes. Thus, it is important to monitor future mutations and their associated biochemical properties to anticipate future drug-resistance.

Michael Dominic Sacco1, Yanmei Hu2, Maura Verenice Gongora2, Flora Meilleur3,4, Michael Trent Kemp1, Xijun Zhang2, Jun Wang2,3 and Yu Chen1

1Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL, USA. 2Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey, Piscataway, NJ, USA. 3Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC, USA. 4Neutron Scattering Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA.

email: junwang@pharmacy.rutgers.edu; ychen1@usf.edu

Fig. 1  Biochemical and structural comparison of WT and Omicron Mpro P132H. a Characterization of enzymatic activity shows that WT Mpro and Omicron Mpro have comparable catalytic activity. b Thermal shift assay for apo proteins demonstrates that Omicron Mpro has a lower Tm than WT Mpro by 2.6 °C. c Time-dependent proteolytic activity suggests that substrate turnover in both enzymes decreases at a similar rate when incubated at 37 °C for extended periods of time. d Covalent inhibitors GC-376, PF-07321332 (nirmatrelvir), and PF-00835231 are equally potent against WT Mpro and Omicron Mpro. e Likewise, by assessing their ΔTm as a function of inhibitor concentration, we show that they are both stabilized to a similar extent by GC-376, PF-07321332 (nirmatrelvir), and PF-00835231. f Crystal structure of SARS-CoV-2 Omicron Mpro + GC-376 at 2.05 Å resolution (green) superimposed with WT Mpro + GC-376 (blue; PDB ID 7C6U). P132H is shown as spheres. g Electron density map of H132 and surrounding residues. 2Fo-Fc map is contoured at 1 σ and shown in gray. h Structural comparison of position 132 and interacting residues in WT Mpro and Omicron Mpro. i Top 25 most common Mpro mutants. j Top 25 most common Mpro mutants mapped onto the crystal structure of SARS-CoV-2 Omicron Mpro as orange spheres. Three most common mutants P132H, K90R, and L89F are labeled.

Cell Research (2022) 32:498 – 500
DATA AVAILABILITY
The X-ray crystal structure of the Omicron M\textsuperscript{RMSD} P132H mutant in complex with GC376 was deposited in PDB with the code 7TOB.

REFERENCES
1. Cannalire, R. et al. J. Med. Chem. https://doi.org/10.1021/acs.jmedchem.0c01140 (2020).
2. Owen, D. R. et al. Science 374, 1586–1593 (2021).
3. WHO. https://www.who.int/en/activities/tracking-SARS-CoV-2-variants Accessed on 19 February 2022.
4. Ma, C. et al. Cell Res. 30, 678–692 (2020).
5. Dabrowska, A. et al. bioRxiv https://doi.org/10.1101/2021.12.21.473268 (2021).
6. Rai, D. K. et al. bioRxiv https://doi.org/10.1101/2022.01.17.476644 (2022).
7. Vangeel, L. et al. Antiviral Res. 198, 105252 (2022).
8. Rosales, R. et al. bioRxiv https://doi.org/10.1101/2022.01.17.476685 (2022).
9. Ulbrich, S. et al. Boorg. Med. Chem. Lett. https://doi.org/10.1016/j.bmcl.2022.128629 (2022).
10. Greasley, S. E. et al. bioRxiv https://doi.org/10.1101/2022.01.17.476556 (2022).

ACKNOWLEDGEMENTS
X-ray data were collected at the X-ray facility supported by the Spallation Neutron Source (Oak Ridge National Laboratory), a DOE Office of Science User Facility. This research was supported by the National Institutes of Health (NIH) (grants AI147325, AI157046, and AI158775) to J.W. We thank Eric M Lewandowski for reviewing this paper.

AUTHOR CONTRIBUTIONS
M.D.S., J.W., and Y.C. conceived the experiments, interpreted the data, and composed the paper. Y.H. performed the biochemical assays and melting temperature experiments. Proteins were cloned and purified by X.Z. Crystal structures were solved and refined by M.D.S. Crystals were grown by M.V.G. X-ray datasets were prepared and collected by M.T.K and F.M.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41422-022-00640-y.

Correspondence and requests for materials should be addressed to Jun Wang or Yu Chen.

Reprints and permission information is available at http://www.nature.com/reprints